Human Tumor Cell Kinetics

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INTRODUCTION

This series of seminars concerns normal and tumor cell kinetics, with particular emphasis on human neoplasia. For a number of years, the Chemotherapy Area of the National Cancer Institute has been interested in cell kinetics, both leukocyte and solid tumor cell kinetics, and it is a pleasure for us to sponsor this series.

The primary objective of the chemotherapy program of the National Cancer Institute is the achievement of improved control or cure of cancer through the development of new agents and the more effective use of old agents. It has become apparent that rapidly growing tumors are more sensitive to cytotoxic agents than the slowly growing tumors, at least in the dosages and schedules currently used. The terms "rapidly growing" and "slowly growing" refer only to the net growth of the tumor. These terms do not tell us anything about the proportion of cells in active proliferation, the conversion of G_0 cells to cells in cycle, the rate of division, the generation time of the dividing cells, or the rate of cell loss. The replication times of tumor cells in the proliferative phase have not yet been determined unequivocally, although generally they seem to approximate the generation times of their normal counterparts.

However, of greater significance is the fact that rapidly growing tumors apparently have a larger fraction of cells in cycle or in active proliferation. Since most of the antitumor agents currently in use affect cells in DNA synthesis or in some other phase of the mitotic cycle, tumors with large proportions of cells in cycle will therefore be more susceptible to treatment with these agents. Acute lymphocytic leukemia and choriocarcinoma in women are examples of this type of tumor and are representative of those malignancies in which impressive progress has been achieved.

On the other hand, the slowly growing tumors have a relatively small proportion of cells in active proliferation and hence would not be expected to show the dramatic response of the rapidly growing tumors to agents affecting cells in cycle. Slowly growing tumors, such as carcinoma of the breast, colon, and lung, where results of chemotherapy have been relatively poor, are characterized by doubling times measured in terms of weeks or months. Cell kinetic data on these lesions are very sparse, but those available suggest that relatively few cells are in DNA synthesis at any one time. Hence, frequent administration of agents affecting cells in this phase or in some other phase of the cycle over a short interval will have minimal effects on the tumor. At the same time, serious damage to rapidly proliferating normal tissues will occur. Theoretically, then, ideal therapy would be so spaced as to achieve maximum antitumor effect, yet permit repair of susceptible normal tissues.

As indicated above, advances in cancer chemotherapy have largely been confined to the rapidly growing tumors with doubling times measured in days. The best responses have been obtained with intensive therapy administered at frequent intervals, often daily. However, just as tumors have varying rates of growth, normal tissues also have different rates of turnover. It is therefore understandable that intensive therapy is associated with adverse effects on normal tissues having high cell division rates, such as the bone marrow and gastrointestinal tract, but yet there is relatively little effect on tissues which replace themselves slowly. Even though remarkable progress has been achieved in treating the rapidly growing tumors, many questions remain unanswered; but there appear to be differences, not well understood, between rapidly growing tumor cell populations and susceptible normal tissues which permit selective beneficial drug effects.

The foregoing discussion suggests that there are sharp divisions between rapidly growing and slowly growing tumors. There is probably a continuous spectrum of tumors from relatively rapid to relatively slow growing, not only among tumors of different morphologic types, but among patients with tumors of the same morphologic type. In fact, in an individual with metastatic disease, varying rates of growth are often observed clinically. Obviously, this situation compounds the difficulties the therapist confronts in treating patients with neoplasia.

Fundamental data concerning cell kinetics and cell cycle characteristics of both normal and neoplastic cells in man are quite sparse. Virtually nothing is known about the rate and magnitude of cell loss from human tumors. The technical problems are difficult, and new techniques are urgently needed that will permit quick assessment of the cell kinetics of a tumor in an individual patient. Fortunately, there is currently a great deal of research interest in these problems and, hopefully, data permitting the more rational use of antitumor agents will soon be forthcoming. This series of seminars is being presented to review the latest developments in this important area of research. Leading investigators in cell kinetics, both leukocyte and solid tumor cell kinetics, have been invited to discuss their most recent studies. The discussants will attempt to relate their findings as far as possible to the clinical problems, so that this series should be of value to the cancer chemotherapists, radiotherapists, pharmacologists, toxicologists, and others concerned in the effort to improve the control of cancer through the use of antitumor agents.

Seymour Perry, M.D.
Moderator

Biochemical Events in the Cell Cycle

Renato Baserga, *Department of Pathology, Temple University Health Sciences Center, Philadelphia, Pennsylvania 19140*

THE cell cycle can be briefly summarized as follows (*1*). Dividing cells go through a series of cyclic changes that can be divided into four phases: 1) the G_1 period between completion of mitosis and the onset of DNA synthesis; 2) the S phase during which the cell replicates its genetic material; 3) the G_2 period between completion of DNA synthesis and mitosis; 4) mitosis in its classic four phases from prophase to telophase. When cells divide, one or both daughter cells can go through the cell cycle again. Others may take an alternative route and become nondividing cells. This is called "terminal differentiation," and since it is not directly related to the subject under consideration, I will not discuss it. There is a third kind of cell in which we may be interested, and this is represented, for instance, by liver cells, which are usually quiescent but are stimulated to divide after partial hepatectomy. Some investigators like to think that these cells are in a special phase of the cell cycle, called the G_0.

My discussion will be limited to: 1) the events that occur in preparation for DNA synthesis (actually the events that precede and seem to control the initiation of DNA synthesis), therefore the G_1 period; 2) the events that precede and seem to control the initiation of mitosis, the G_2 period; and 3) the events that occur between application of the stimulus and the onset of DNA synthesis in those tissues that can be stimulated to enter DNA synthesis and mitosis.

I will illustrate some of these biochemical events with some of our own experiments, rather than go through the entire literature, which has been adequately covered elsewhere (*2*). Text-figure 1 shows an experiment that was done a few years ago (*3*) with Ehrlich ascites tumor cells growing in the peritoneal cavity of mice. This was an asynchronously growing population in which about 50% of the cells were in DNA synthesis at any given time. Actinomycin D was given in small doses (0.016 μg/g body weight), the doses of actinomycin D first used by Lieberman and co-workers (*4*) to study nucleic acid metabolism. The animals were killed at various intervals after the first injection of actinomycin D and 30 minutes after the injection of tritiated thymidine (^{3}H-TDR) to label DNA-synthesizing cells.

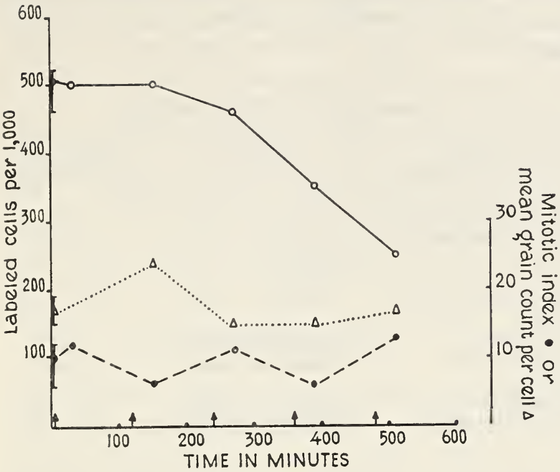
Three things are noticeable in text-figure 1. First, nothing happens to the mitotic index. It fluctuates at random around the mean value of controls, which means that these doses of actinomycin D had no effect on the flow of cells from S phase to mitosis. Second, the percentage of labeled cells remains constant for about 2½ hours and then decreases slowly to reach a low value of about 50% of controls at about 500 minutes after the first injection of actinomycin D. Third, the dotted line represents the mean grain count of labeled Ehrlich ascites cells. It shows that the intensity of the label in cells still synthesizing DNA is not affected by actinomycin D. If actinomycin D inhibited DNA synthesis directly, one would expect an immediate drop in the percentage of labeled cells and a decrease in the mean grain count. The results in text-figure 1 suggest that instead of inhibiting DNA synthesis directly, the entrance of cells into the S phase is inhibited. To demonstrate this point, the experiment described in table 1 (*5*) was performed.

TABLE 1.—Effect of actinomycin D on the percentage of Ehrlich ascites cells labeled by two pulse exposures to ^{3}H-TDR*

	Labeled cells per 1,000	
	After one injection	After two injections
Controls	550 (520–580)	708 (685–720)
Actinomycin D injected	545 (490–600)	588 (505–650)

*Tumor-bearing mice were given 10 μc of ^{3}H-TDR, and a sample of tumor was aspirated from the peritonea cavity after 30 minutes. After another 30 minutes, half the animals were given actinomycin D (0.016 μg/g body weight); injections were repeated every 2 hours for a total of four. One hour after the last injection of actinomycin D, all mice were given another injection of 10 μc of ^{3}H-TDR, and killed after another 30 minutes. Smears for autoradiography were prepared from each mouse after the first injection ofradioisotope and again after killing (from Baserga *et al.*, 1965) (*5*).

Ehrlich ascites tumor cells growing in the peritoneal cavity of mice were given an initial injecton of ^{3}H-TDR which resulted in the labeling of about 55% of the cells. If the animals were left untreated and then given a second injection of ^{3}H-TDR 8 hours after the first, new cells that were in G_1 at the time of the first injection had by that time proceeded into the S phase. When the second injection was given, the percentage of labeled cells increased from 55 to about 70%. Another group of animals, after receiving the first injection of ^{3}H-TDR were treated with actinomycin D in low doses, 0.016 μg/g body weight, every 2 hours. Then they received a second injection of ^{3}H-TDR. Table 1 shows that the percentage of labeled cells increased slightly but certainly not as much as it did in control mice. This indicates a block in the flow of cells from G_1 into S phase. The finding that this block was produced by actinomycin D suggests that RNA synthesis may be involved, but for the moment it can be referred to as an actinomycin D-sensitive step.



TEXT-FIGURE 1.—Effect of repeated injections of actinomycin D (0.016 μg/g body weight) on the fraction of cells in DNA synthesis in Ehrlich ascites tumor growing in the peritoneal cavity of mice. *Arrows* indicate times at which actinomycin D was given: ^{3}H-TDR was given 30 minutes before killing. ○——○ labeled cells per 1,000; ●- - -● mitoses per 1,000 cells; △...△ mean grain count per labeled cell. Each value is the mean of at least 3 animals; *bars* indicate range of control values [from (*3*)].

If there is such a step, possibly there are puromycin or cycloheximide-sensitive steps elsewhere. This was shown in an experiment done by Terasima and Yasukawa (*6*), in which they exposed synchronized cells in tissue culture for 2 hours to either cycloheximide or puromycin, both inhibitors of protein synthesis. In untreated animals, as the cells progressed from G_1 into the S phase, ^{3}H-TDR was incorporated into DNA very rapidly. The same thing happened in treated cells, but there was a 2-hour delay, regardless of the time of addition of the cycloheximide or the puromycin. Apparently there is a step in G_1 (probably protein synthesis) that is sensitive to either cycloheximide or puromycin, and when this step is inhibited, the entrance of cells into DNA synthesis is correspondingly delayed. A third feature of the G_1 period may be of interest. Thymidine kinase activity decreases during the G_1 period (*7*, *8*), while another enzyme of DNA synthesis, DNA polymerase, remains elevated throughout the various phases of the cell cycle [*see* review in (*2*)].

A little more is known about the G_2 period, the interval when the cell prepares for actual division (mitosis). What we know in this respect is summarized in table 2, which presents the events in the G_2 period that can be related to the control of mitosis. It is well known that during the G_2 period protein synthesis in general decreases. Kishimoto and Lieberman (*9*) were the first to demonstrate periods in the G_2 phase which are sensitive to either actinomycin D or puromycin. The results of these experiments have been confirmed by many others in a variety of tissue culture systems. Also in the G_2 period there are steps sensitive to cycloheximide and mengovirus (*10–12*). An infection with mengovirus promptly produces an inhibition of protein synthesis. Thus inhibitors of protein synthesis applied during the G_2 period produce a G_2 block. However, if one waits until the very late G_2 period before exposing cells to these inhibitors of protein synthesis, a block in metaphase results. This means that the proteins necessary for the cell to go through the G_2 period have already been produced so that the cell may progress to mitosis; but it is then unable to go beyond metaphase.

TABLE 2.—Events in G_2 related to the control of mitosis*

Time period	Biochemical event	Effect	Reference
G_2	Decreasing protein synthesis		*(13)*
Early G_2	Step sensitive to actinomycin D.	G_2 block	*(9, 11)*
G_2	Steps sensitive to puromycin, cycloheximide, and mengovirus.	G_2 block	*(9, 10–12)*
Very late G_2	Puromycin-sensitive	Metaphase block	*(11)*
G_2, prophase	*p*-Fluorophenylalanine-sensitive step.	Metaphase delay	*(14)*
G_2	Vinblastine-sensitive step	Metaphase block	*(15, 16)*
G_2	Inhibition by alkylating agents.	G_2 delay or block	*(17, 18)*

*From *(2)*.

Another interesting experiment was done by Sisken *et al.* *(14)*, using *p*-fluorophenylalanine, which does not inhibit protein synthesis *per se* but is incorporated into protein and forms, so to speak, fraudulent proteins. When these workers *(14)* applied *p*-fluorophenylalanine to cells in G_2 or in prophase, it produced a metaphase delay. The metaphase, instead of lasting only 20 minutes, lasted 2–3 hours, suggesting that the defective protein made during G_2 or prophase in the presence of *p*-fluorophenylalanine made it difficult for the cells to complete mitosis. This was shown by time-lapse cinematography, and the experiments seem to be quite convincing. Vinblastine also produces a metaphase block when applied during the G_2 period *(15, 16)* Finally, one can also produce a G_2 delay or block with alkylating agents, as first shown in our laboratory a few years ago by Layde and myself *(17)* with nitrogen mustard.

Let's return to our scheme of the cell cycle and consider the cells which, as we have said, some investigators put in this "limbo" called G_0. Cells in this phase are quiescent but can be stimulated first to enter DNA synthesis and then mitosis if a proper stimulus is applied. There are several of these models which I call models of stimulated DNA synthesis; these are summarized in tables 3, 4, and 5.

Table 3 lists the *in vitro* models of stimulated DNA synthesis. The classic one is the primary culture of kidney cells. Primary explants of kidney cells were first studied by Lieberman and his co-workers *(4)*. They showed that if rabbit kidney cells are explanted, they go through a lag period and then after 30–50 hours (the prereplicative period) enter DNA synthesis. Since then, several other systems have been described; for instance stationary cell cultures can be induced to synthesize DNA by simian virus 40 or polyoma virus *(19–22)*. A typical example is the one described in detail by Cooper *et al.* *(23)*, in which lymphocytes in blood culture are stimulated to enter DNA synthesis by phytohemagglutinin and then divide. The hybridization of hen erythrocytes is an interesting system. In the experiments described by Harris (*24*), use was made of the hen erythrocyte, a nucleated cell in which the nucleus does not go into DNA synthesis and the cell does not divide. If these nondividing nucleated hen erythrocytes are hybridized with HeLa cells, the nucleus of the hen erythrocyte is stimulated to enter DNA synthesis. Another system described by Todaro *et al.* (*25*) consists of the stimulation of contact-inhibited cells in culture by the addition of a small amount of dialyzed serum. Approximately 6% of the cells enter into DNA synthesis in about 14–15 hours. Then there is the stimulation of organ cultures of skin by the epidermal growth factor, a protein isolated in purified form by Cohen (*26*). The introduction of epidermal growth factor in an organ culture of skin "induces" DNA synthesis. Finally, there are the experiments of Topper and his co-workers (*27*) on the stimulation of DNA synthesis and cell division by insulin in explants of mouse mammary glands. These are the *in vitro* models. There is also a series of *in vivo* models and, of course, since I have always worked more with animals than with tissue cultures, I am somewhat prejudiced in favor of the *in vivo* models.

TABLE 3.—*In vitro* models of stimulated DNA synthesis and cell division*

System	Stimulus	Length of prereplicative period	Reference
Stationary cell cultures	Simian virus 40	20 hr	(*19, 20*)
Stationary cell cultures	Polyoma virus	16–20 hr	(*21, 22*)
Lymphocytes in blood cultures	Phytohemagglutinin	24 hr	(*23*)
Hen erythrocyte	Hybridization	—	(*24*)
Contact-inhibited cells in culture	Dialyzed serum	14–15 hr	(*25*)
Primary cultures of kidney cells	Explant	30–50 hr	(*4*)
Organ cultures of skin	Epidermal growth factor.	2 days	(*26*)
Explants of mouse mammary gland.	Insulin	—	(*27*)

*From (*2*).

Table 4 shows some of the *in vivo* models of stimulated DNA synthesis and cell division. Stimulation of DNA synthesis in liver after partial hepatectomy, either in rat or mouse, is widely known. An interesting system described by Steiner and co-workers (*28*) is the stimulation of DNA synthesis by insulin in the liver of the alloxan-diabetic rat, again with a prereplicative period of 24 hours. DNA synthesis in the salivary gland can be stimulated by a single administration of isoproterenol (IPR). I will come back to this later. This was shown first by Barka (*29*) in the rat, and we subsequently confirmed it in the mouse (*30*). Unilateral nephrectomy results in an increase in DNA synthesis and cell division in the contralateral kidney, but much more effective is the single administration of folic acid which causes a great increase in DNA synthesis in the kidney of both rat and mouse (*31*). According to Stöcker (*32*), temporary ischemia of the kidney also produces an increase in DNA synthesis in the kidney. Approximately 93% of kidney cells are stimulated to enter DNA synthesis. Lotspeich (*33*) showed that metabolic acidosis produces DNA synthesis in rat kidney, and Cuppage and Tate (*34*) reported that injury with mercuric chloride can also produce proliferation of kidney cells. Growth hormone can induce DNA synthesis, always associated with lag period, in the rib cartilage of the hypophysectomized rat (*35*).

TABLE 4.—*In vivo* models of stimulated DNA synthesis and cell division*

Tissue or organ (animal)	Stimulus	Length of prereplicative period	Reference
Liver (rat, mouse)	Partial hepatectomy	18 hr	(*36, 37*)
Liver (alloxan-diabetic rat)	Insulin	24 hr	(*28*)
Salivary gland (rat, mouse)	Isoproterenol	20 hr	(*29, 30, 38*)
Kidney (rat, mouse)	Controlateral nephrectomy	1–2 days	(*39*)
Kidney (rat)	Folic acid	1–2 days	(*31*)
Kidney (rat)	Temporary ischemia	20–30 hr	(*32*)
Kidney (rat)	Metabolic acidosis	—	(*33*)
Kidney (rat)	Mercuric chloride necrosis	1–3 days	(*34*)
Cartilage (hypophysectomized rat)	Growth hormone	1–2 days	(*35*)

*From (*2*).

Table 5 shows additional systems of stimulated DNA synthesis. Probably the most interesting are those in which estrogens are used to stimulate DNA synthesis in target organs, especially the uterine epithelium and the mammary glands (*40*, *41*). There has been a great deal of study on the early events that follow the interaction of estrogens with target organs. Other systems are perhaps more a curiosity than really valuable for this kind of study. These include regeneration of the lens epithelium after mechanical injury (*42*), regeneration of the pancreas after ethionine (*43*), and the effect of erythropoietin on mouse spleen (*44*). By itself stands the repair of the skin after wounding, in which the prereplicative period instead of lasting the usual 18–20 hours, lasts only 1 hour. This is one of the puzzles in our little scheme and for the moment it remains unsolved.

I have chosen as an illustration the system I have been working with lately, that is, the IPR-stimulated salivary gland of mice. IPR is a catecholamine with the same structure as epinephrine except that the methyl group at the end of the side chain has been replaced by a bulky isopropyl group. IPR can be obtained in large amounts; it is a relatively inexpensive compound and is chromatographically pure. When a single injection of this compound of 1 μmole/g body weight is given to mice, and the incorporation of ^{3}H-TDR into DNA is determined 30 minutes after exposure to thymidine, the specific activity of DNA at various intervals after IPR follows the pattern we described in a previous paper (*30*).

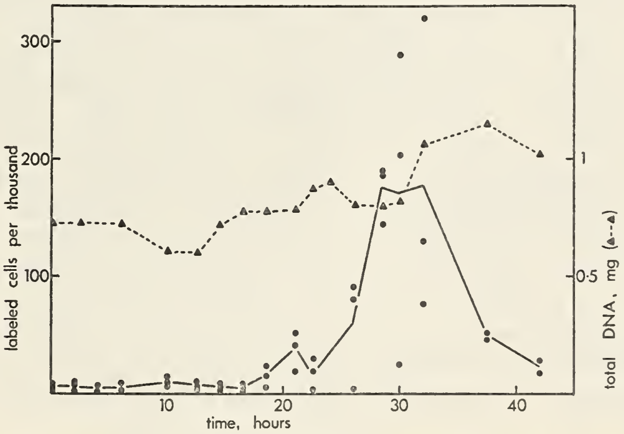
TABLE 5.—*In vivo* models of stimulated DNA synthesis and cell division*

Tissue or organ (animal)	Stimulus	Length of prereplicative period	Reference
Uterine epithelium (mouse)	Estrone	—	*(41)*
Mammary gland (mouse)	Estradiol-progesterone	3–4 days	*(40)*
Mammary gland (mouse)	Lactation	1–2 days	*(45)*
Lens epithelium (rabbit)	Mechanical injury	14 hr	*(42)*
Pancreas (rat)	Ethionine	2–3 days	*(43)*
Skin (mouse, rat)	Wounding	1 hr	*(46)*
Spleen (mouse)	Erythropoietin	18 hr	*(44)*

*From *(2)*.

Nothing happens for about 18 hours, the usual prereplicative period, and then DNA synthesis begins to increase, reaching a level approximately 25-fold the control values. Then it decreases and by 44 hours it is back to control levels; the cells are again quiescent.

The same experiment is shown in text-figure 2, but instead of the specific activity of DNA we have the percentage of labeled cells (labeled cells/1,000), and here again nothing has happened for 18 hours. Then the cells begin to get "restless" at about 20 hours and the fraction of labeled cells increases dramatically. These cells become quiescent again by 42 hours, but there is a net synthesis of DNA.



TEXT-FIGURE 2.—Effect of a single injection of IPR (1.0 μmole/g body weight) on DNA synthesis in mouse salivary glands. The *abscissa* represents time in hours after IPR. Mice were given ^{3}H-TDR 30 minutes before killing; ●——● labeled salivary gland cells per 1,000; ▲---▲ total DNA per gland (mean of 3 values) [from *(30)*].

How many salivary gland cells are actually stimulated to enter DNA synthesis by a single administration of IPR? This is shown in table 6. In the parotid gland of control mice, after a single injection of ^{3}H-TDR, about 1 cell per 1,000 can be labeled. When ^{3}H-TDR is injected repeatedly every 3.5 hours for 9.5 hours, 3 cells per 1,000 can be labeled. When parotid cells are stimulated with IPR, 61% of them go into DNA synthesis, as shown by labeling with repeated injections of ^{3}H-TDR. After a second injection of IPR (which we will come to soon) as many as 80% of the parotid cells can be stimulated to enter DNA synthesis.

TABLE 6.—Percentage of mouse parotid cells labeled by ^{3}H-TDR after IPR*

Treatment	^{3}H–TDR	% Labeled cells
None	Pulse-label†	0.1 (0.07–0.1)
None	Repeated injections‡	0.3 (0.1–0.5)
IPR once	Pulse-label†	22.3 (20–26)
IPR once	Repeated injections‡	61.3 (52–74)
IPR twice	Pulse-label†	41.0 (19-51)
IPR twice	Repeated injections‡	77.5 (74–80)

*IPR (1.0 μmole/g body weight) was given intraperitoneally; when given twice, the two injections were 48 hours apart. There were 3 animals per group.

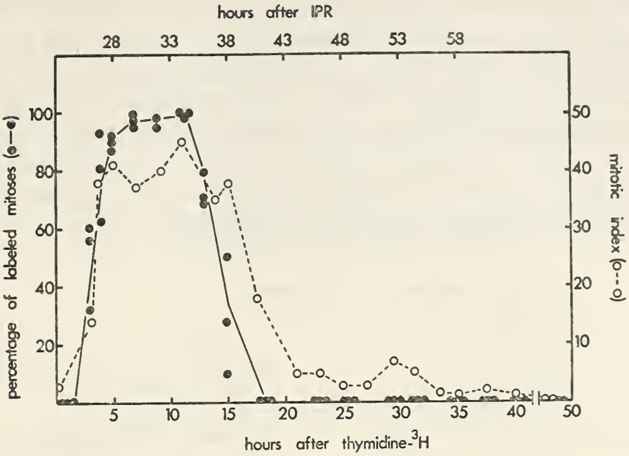
†Pulse-label of ^{3}H-TDR at 25 hours (1 injection of IPR) or 21 hours (2 injections) after IPR.

‡Repeated injections of ^{3}H-TDR covering a span of 9.5 hours, beginning at 22 hours with one injection and at 18 hours with 2 injections of IPR. ^{3}H-TDR 10 μCi per mouse subcutaneously was given every 3.5 hours.

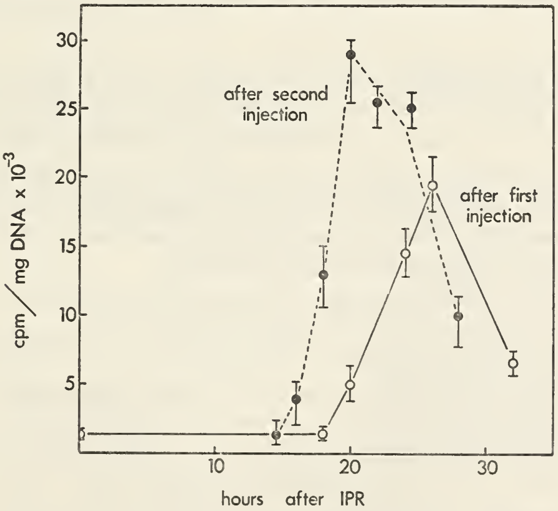
The cell cycle of the parotid cells stimulated by IPR is shown in text-figure 3. To determine the cell cycle, mice were given IPR and 23 hours after its injection ^{3}H-TDR was given. The animals were killed at different intervals after injection of ^{3}H-TDR, and the percentage of labeled mitoses was determined by autoradiography (*closed circles*). There is a G_2 period of 3 hours and an S phase of 9 hours, but the most interesting part is that the cells divide only once. They do not go back into the cycle without further stimulation. It is apparent that the mitotic index correlates with the peak of DNA synthesis (text-fig. 2). Unquestionably we are dealing here with *bona fide* induction of cellular proliferation. It is not only a question of increased incorporation of thymidine but increased incorporation of thymidine followed by cell division and net increase in DNA. Eighty percent of these cells are stimulated to divide, and they divide only once. This is a useful system, since a purified chemical compound is being used, one injection is given, and the response does not require the services of a statistician to be considered significant. We have been trying to identify the series of biochemical events that lead to the onset of DNA synthesis.

Text-figure 4 shows what happens after a second injection of IPR. I said that after the first injection of IPR there is a good DNA-synthesis response beginning at approximately 20 hours and peaking at about 28 hours. If one waits about 48 hours after the first injection and then gives a second shot of IPR, when the cells are all quiescent, the cells go back to DNA synthesis. They now begin at 16 hours and reach an even higher peak of DNA synthesis.

We first tried to study RNA and protein synthesis with this model, but decided the most urgent thing to do in a system such as this was really

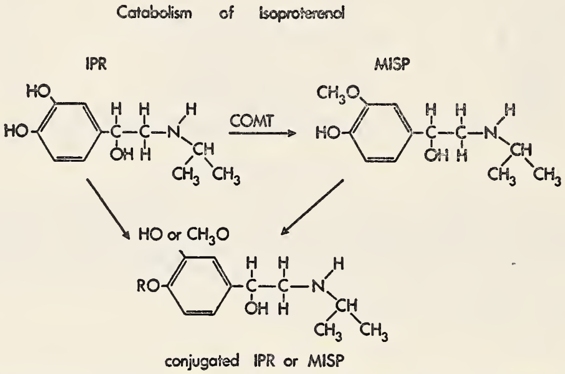


TEXT-FIGURE 3.—Percentage of labeled mitoses in parotid at various times after a single injection of ^{3}H-TDR (given 23 hours after a single injection of IPR, 1.0 μmole/g body weight). *Note* only one wave of labeled mitoses. Each *closed circle* (percentage of labeled mitoses) represents one animal; *open circles* (mitoses/1,000 cells) are the average of 3 mice per point.



TEXT-FIGURE 4.—Effect of one or two injections of IPR (1.0 μmole/g body weight) on DNA synthesis in mouse salivary gland. ^{3}H-TDR (10 μCi/mouse, subcutaneously) was given 30 minutes before killing. The second injection of IPR was given 48 hours after the first. The *ordinate* gives the specific activity of mouse salivary gland DNA.

to try to follow IPR. What happens to this compound? Where does it go? Where does it bind? Again, we were lucky because IPR has only two catabolites in the animal body. It is either methoxylated on carbon 3 of the phenol ring producing 3-methoxyisoproterenol, or it is glucuronized either as unchanged IPR or as the methoxy derivative (text-fig. 5). Thus by a simple chromatographic procedure one can determine the amount of IPR present in various tissues either as unchanged IPR, or as methoxyisoproterenol, or as glucuronized IPR.



Text-figure 5.—Isoproterenol and its catabolites. IPR = isoproterenol; COMT = catechol-O-methyl transferase; MISP = 3-methoxyisoproterenol; R = glucuronic acid.

One of the first things we found was that when we injected an inducing dose of IPR, part of which was tritiated, a lot of radioactivity was found in the liver, five times more than in the salivary glands, even when expressed as per gram of wet tissue. However, in the salivary gland 47% of the total radioactivity was as unchanged IPR, whereas in the liver it was less than 10%. When we did detailed experiments with cell fractionation, the results were even more interesting.

Table 7 shows the percentage of the total radioactivity in the nuclei, microsomal fraction, and supernatant of either salivary gland or liver 30 minutes after an inducing dose of IPR. In the nuclei of the salivary gland and also in the other fractions, there was a large percentage of unchanged IPR, whereas in the liver most of it was glucuronized. Many experiments have shown that the action of IPR on the salivary gland may be direct [*see 38*]; that is, it is not mediated through other organs. In another experiment we gave a single injection of IPR and determined the incorporation of tritiated uridine into salivary gland RNA; instead of getting the increase that everybody else gets with older models we actually got a decreased incorporation of uridine into RNA (*47*). With time, the incorporation of uridine increases to about twice the control value. But when we did a carefully balanced study and determined the pool size of uridine phosphate in salivary glands at various intervals after IPR, we found the UTP was decreased at the beginning and increased at around 20 hours. In fact, one of my postdoctoral fellows, Dr. Malamud, has recently found that the decrease is due to decreased activity of uridylate kinase. Thus when we balanced out specific activity of RNA and specific activity of precursor pool, a flat curve was obtained. There is no decrease or increase in RNA synthesis after IPR, at least between 2 and 24 hours.

TABLE 7.—Distribution of IPR and 3-methoxyisoproterenol (MISP) in subcellular fractions of mouse salivary gland and liver*

	Salivary gland	Liver
Percent of radioactivity in nuclei:		
IPR	68	4. 5
MISP	2	1. 5
Percent of radioactivity in microsomes:		
IPR	45	5. 5
MISP	6	4. 2
Percent of radioactivity in S_3:		
IPR	38	5. 6
MISP	8	5. 5

*Six mice given intraperitoneal injections of 100 μCi of ^{3}H-IPR plus 6 mg of nonradioactive IPR and killed after 30 minutes. IPR and MISP were isolated from the various fractions by chromatography.

However, we obtained interesting results when small doses of actinomycin D (0.016 μg/g body weight) were used. At this level there was no effect on DNA synthesis *per se*, no immediate effect on protein synthesis, and no effect on respiration. When these small amounts of actinomycin D were used, the stimulation of DNA synthesis was effectively inhibited (*47*). The results are summarized in table 8.

TABLE 8.—Effect of actinomycin D (0.016 μg/g body weight) on IPR-stimulated DNA synthesis in mouse salivary glands*

Treatment	cpm† DNA/mg DNA—27 hr after IPR injection
None	2, 100 (1, 500–2, 500)
IPR only	24, 000 (16, 000–32, 000)
Actinomycin D only	2, 100 (1, 400–3, 000)
Actinomycin D 20 min before IPR	300 (150–400)
Actinomycin D 2 hr after IPR	2, 000 (400–3, 700)
Actinomycin D 6 hr after IPR	3, 000 (300–7, 500)
Actinomycin D 24 hr after IPR	16, 000 (3, 800–22, 000)

*From (*47*).
†cpm=Counts per minute.

Another interesting investigation was done with cycloheximide, which has a very short period of action in the salivary gland. It inhibits protein synthesis for only 3–4 hours and after 4 hours the inhibitory action wears off. Table 9 shows what happened when this inhibitor was used. The control animals were given IPR, 6 mg/mouse, and killed at various hours after IPR injection, always with ^{3}H-TDR 30 minutes before killing. We had high specific activities in the IPR controls only, but if we gave cycloheximide 30 minutes or 1 hour or 8 hours before IPR, the DNA synthetic response was practically abolished, especially at 1 hour. Yet the cell was not synthesizing protein for only 4 hours, suggesting that at 1 hour after IPR injection or at 8 hours after IPR a protein is made. The template that makes this protein has a limited half-life, so that if the cell can be kept from making this protein for 4 hours, the template decays and we do not get any DNA synthesis later, even at very late hours. If cycloheximide is present at 24 hours when the templates and all the enzymes for DNA synthesis have been made, then DNA synthesis increases, even above control values.

TABLE 9.—Effect of cycloheximide (CX) on IPR-stimulated DNA synthesis in mouse salivary gland*

Hours after IPR injection (6 mg/mouse)	IPR only	cpm† DNA/mg DNA (% of IPR only) (time of administration of CX before or after IPR)				
		−30 min	+1 hr	+8 hr	+20 hr	+24 hr
23	9, 000	24	1	13	16	
25	11, 000	7	1	35	21	
27	9, 000	13	1	59	25	
30	9, 000	21	1	28	62	59
33	6, 000	36	15	44	73	142

*All mice given ^{3}H-TDR 30 minutes before killing. Values in "IPR only" column are actual cpm/mg DNA; all other values are percent of IPR only.

†cpm = Counts per minute.

These are some of the advantages of IPR in the sense that here is a controllable system in which some of the processes leading to the onset of DNA synthesis can be studied.

Obviously we do not have a detailed description of the biochemistry of the cell cycle. We have only a crude idea of some of the preparative steps necessary for the cells to enter DNA synthesis and to divide. At least now, however, we seem to have the problem within boundaries, and it is possible to pursue it in more detail.

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Dynamics of Growth in Tumors and in Normal Organisms[1]

ANNA KANE LAIRD, *Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439*

MY discussion of the mathematical analysis of tumor growth falls into 3 sections: 1) the mathematical properties of the Gompertz equation, with more emphasis on those properties that may yield insight into biological aspects of growth, rather than on those that the mathematician would wish to emphasize; 2) a discussion of radioautographic studies of tumor cell kinetics, as reported by experts in that area, in an effort to relate these findings to the biological properties of overall tumor growth as defined by use of the Gompertz growth equation; 3) a brief review of several studies bearing on the physiological mechanisms that might be responsible for the observed retardation of tumor growth. Finally, I shall present a conjecture of my own that would explain the retardation of both tumors and normal growth as a consequence of the same underlying biological phenomenon.

GOMPERTZ GROWTH EQUATION

GOMPERTZ CURVE

Our growth equation is based on the empirical observation that the specific growth rate of tumors, as well as of normal organisms and their parts, undergoes a rapid decay during growth (*1*). This decay is closely approximated by an exponential curve or a straight line on a semilog plot. That is,

$$log\left(\frac{d}{dt} log\ W\right) = log\ A_o - \alpha t \qquad [1]$$

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[1] Research supported by U.S. Atomic Energy Commission.

where A_o is the initial specific growth rate, α is the proportional rate of decay of A_o, and W is the mass of the tumor in any units. When this expression is integrated, we obtain

$$W(t) = W_o e^{\frac{A_o}{\alpha}(1 - e^{-\alpha t})} \qquad [2]$$

where $W(t)$ is the mass at time t, W_0 and A_0 are the initial mass and the initial specific growth rate for the period of observation, and α, as before, is the rate of exponential decay of A_o.

This Gompertz growth equation is an asymmetric sigmoid curve, with inflection point at 0.37 of the asymptote. Because it is a single-valued function of t that goes to an asymptote, this curve has the following mathematical properties that are of significance in providing us with information about the biological systems that are fitted by it:

1) Every point on the curve bears a unique relation to the asymptote (whose value can be calculated very simply). Therefore, the properties of growth can be precisely defined for any mass W, whether observed or extrapolated. For example, the instantaneous absolute or specific growth rate can be calculated for any tumor size or any time t, thus allowing valid comparisons for the first time of the growth rates of two different tumors, or of the same tumor grown under different conditions.

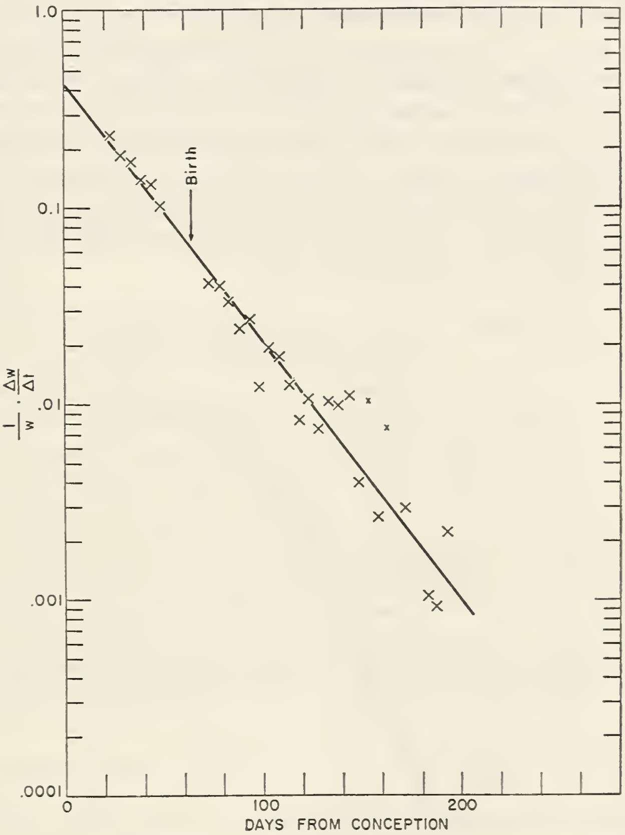
2) If we can define uniquely and exactly any point in its relation to the whole pattern, then we can so define any two points and we then can thus determine the relative time required for growth to pass from one of the two pairs of corresponding points to the other. A useful example would be the time required in a given pattern of growth for the tumor to grow from one-half its size at the inflection point to the size at the inflection point; this time will be long if the curve is flat and short if the curve is steep. This has proved valuable in analyzing the growth of the fetus, where ongoing morphogenetic changes define "stages" of growth, and we would like to make valid comparisons of the part of the pattern required in different species for the same sequence of events. Expansion or contraction of the time scale could prove important in the study of tumors if, for example, certain antitumor agents should slow tumor growth in this way.

3) Because the pattern of the growth curve has its own time scale, while we as observers have our own, it becomes necessary to translate from one time scale to the other. For example, we must learn to think when birth occurs on the organism's own growth pattern, rather than fixing birth by calendar time alone. With tumors, the timing of tumor inoculation or of antitumor therapy should be identified in terms of the growth pattern.

4) The specific growth rate is always decaying. Hence, although the integral equation has an inflection point, at which the absolute growth rate (*e.g.*, daily or weekly) reaches a maximum value and then declines,

the inflection point has no special significance at all in terms of the ongoing decay of the specific growth rate, *i.e.*, of exponential growth. This point is illustrated in text-figure 1, a semilog plot of the specific growth rate during prenatal and postnatal growth of the guinea pig [reprinted from an earlier study (*1*)].

5) The ratio of A/α defines the position of any point on the growth pattern, regardless of the actual values of A or α. Thus, if the initial specific growth rate (A_o) is large in relation to its rate of exponential



TEXT-FIGURE 1.—Changes in the specific growth rate with time, for the prenatal and postnatal growth of the guinea pig. The specific growth rate was calculated by dividing the difference between successive body weights by the time interval between these points, the whole fraction being divided by the mean body size for that interval, defined as equal to the earlier size plus two-thirds the difference between the successive values. Each point was then plotted at a time equal to the earlier time plus two-thirds of the interval between the successive measurements.

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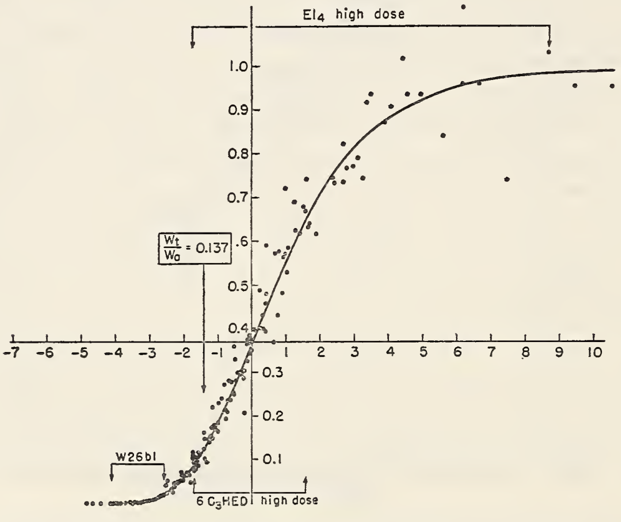
decay (α), the growth measurements will lie far to the left of the inflection point, and the growth curve of that tumor will bear a moderately close resemblance to an exponential curve throughout its growth. But if the value of A_0 is very low in comparison with α, the growth measurements will approach the asymptote. Between these two extremes, the growth data will lie mainly in the region just before the inflection point and by chance will also fit a cube-root curve. Therefore, the relatively common "cube-root growth" is due to a fortuitous balance between the specific growth rate and its rate of decay.

Text-figure 2 is an illustration from an earlier study (2), which included the growth data of all the tumors in the literature at that time. The data have been normalized for this text-figure: the ordinate by dividing each tumor measurement by the asymptote of its own curve, and the abscissa by multiplying the time of each tumor measurement (in days) by the value of α obtained on fitting the growth curve to the data. The curves were superimposed at the inflection point, and the time required for the doubling in tumor size immediately preceding the inflection point was arbitrarily defined as one standard time unit.



TEXT-FIGURE 2.—A "normalized" Gompertz plot, in which the growth data for 19 examples of 12 different tumors of the rat, mouse, and rabbit have been superimposed after adjustment of the units on the 2 axes. The point of reference, at the intersection of the 2 scales, is the inflection point of the growth curve. The units on the ordinate (tumor size) are decimal fractions of the asymptotic tumor size. The unit of time on the abscissa is the time required for the doubling immediately preceding the inflection point (extending from −1 to 0 in this text-fig.).

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Because this sample includes different types of tumors (carcinomas and sarcomas, ascites and solid tumors) and hosts (three species), the pattern of growth defined by the Gompertz equation appears to be a general biological characteristic of tumor growth. Within that pattern, each tumor occupies only a small but characteristic range of growth. However, the sample as a whole covers the entire possible range of Gompertzian growth, from one tumor of the rat farthest to the left of the inflection point, whose decay of the specific growth rate was not statistically different from zero (*i.e.*, it approximated simple exponential growth), to certain mouse tumors whose growth passes through the inflection point and approaches a final limiting size.

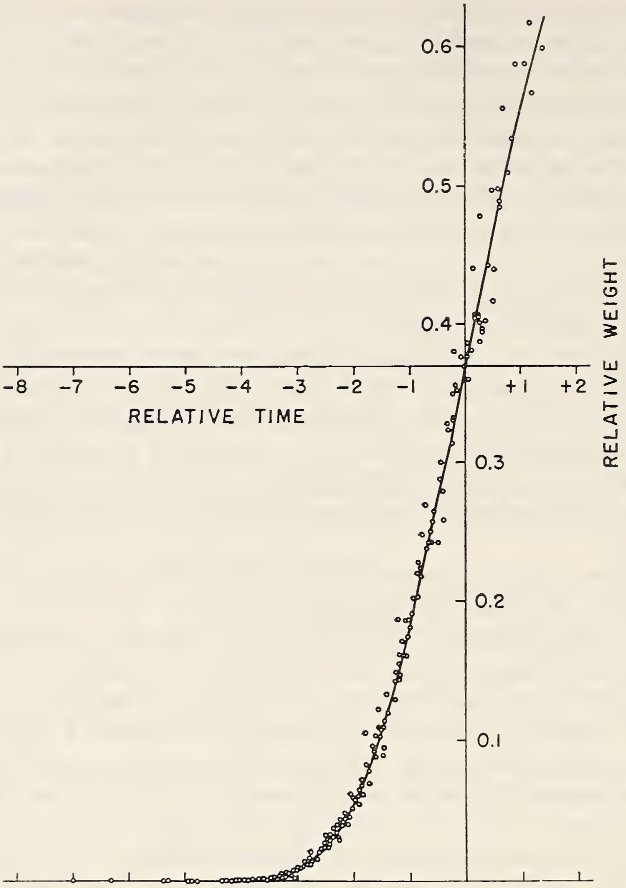
In general, the rat tumors are far to the left in text-figure 2, and the mouse tumors to the right, with little overlap. Since the position of any point, or set of points, on the pattern is determined, as we noted above, by the ratio of the specific growth rate to its rate of exponential decay, the demonstration of a species differential in the position of the tumors on the pattern of text-figure 2 indicates that this is a biologically meaningful relation.

Text-figure 3 is a similar normalized plot of the growth of the fetuses of a number of species of birds and mammals, taken from a previous study (*3*). This is also a Gompertz curve, and the individual growth measurements of each species were normalized in exactly the same way as for text-figure 2. We can draw two conclusions immediately: 1) A similar unity of growth pattern exists among the fetuses of a wide variety of animal species (this text-fig. includes all the data in the literature, so far as I am aware; 15 species are represented, including 3 mammalian and 4 avian Orders); and 2) the same dynamic pattern of growth is shared by normal fetal growth and tumor growth. However, one difference exists between fetal and tumor growth: All fetal growth falls within a single segment of a Gompertz curve, whereas our sample of tumors is already varied enough to include the entire possible range of Gompertzian growth.

In addition, normalization of the fetal curves showed that all these species were conceived at about the same time and were born at about the same time on the standard curve; small differences in timing corresponded to the degree of maturity at birth and to differences in the time at which the embryo first exceeded the original size of the fertilized ovum. These findings strengthen our confidence that analysis of growth using this equation does indeed yield biologically meaningful comparisons of growing systems.

SPECIFIC GROWTH RATE CURVE

The sigmoid curve we have been considering is plotted on a linear scale, but it is describing exponential changes on both scales, a fact which makes this curve difficult for the unwary to interpret. So let us turn to text-figure



TEXT-FIGURE 3.—A "normalized" Gompertz plot in which the growth measurements of the embryos of 15 species of birds and mammals have been superimposed after adjustment of the units on the 2 axes. The procedure of normalization is identical to that shown in text-figure 2.

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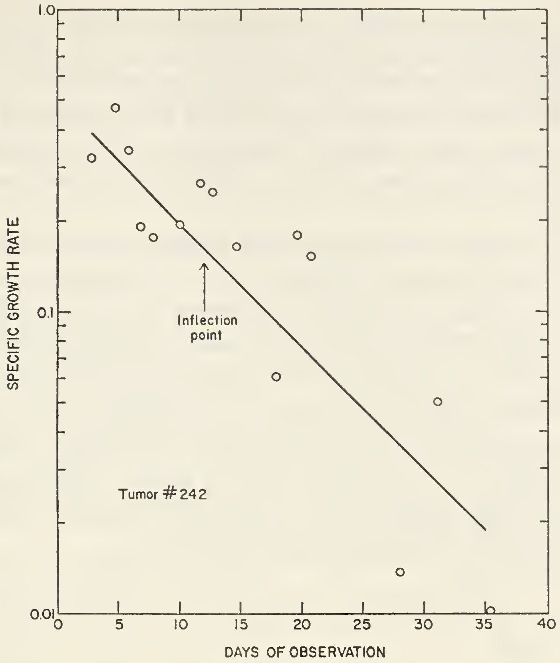
4, a semilog plot of the specific growth rate of a tumor against time. The individual points were calculated by dividing the increase in tumor size between successive points by the time between the same two points, this fraction then being divided by the weighted mean tumor size for that tumor interval. The time for each point was taken as the weighted mean time of the interval. Such a plot exaggerates the variance of the data considerably, because it is based on the differences between points. But it serves our purpose here, which is only to illustrate the Gompertz growth model. The scatter results in a band of points, which appears to be linear, paralleling and enclosing the calculated least-squares line. Use of the

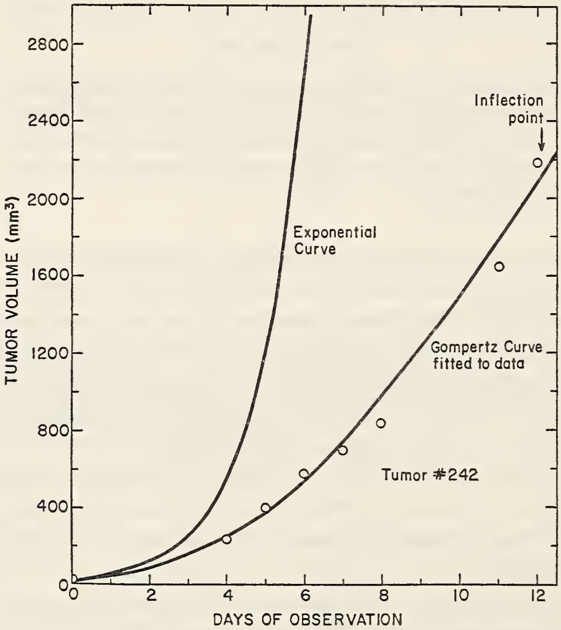


Text-figure 4.—Semilog plot of the changes undergone by the specific growth rate of a tumor with time. The tumor is a transplantable mammary carcinoma grown in a male C3H mouse. Calculation as for text-figure 1.

Gompertz equation depends on individual interpretation at this stage, but only at this stage: If one believes that this pattern of points is well interpreted as a straight line, then growth of tumors (and of other normal systems as well, such as the growth of the guinea pig shown in text-fig. 1) is necessarily correctly represented by a Gompertz equation, because this equation is the integrated form of the differential equation represented by the straight line in text-figures 1 and 4. The various properties of the Gompertz equation referred to in earlier paragraphs are also correctly stated properties of tumor growth (and of normal growth) because they are merely verbal representations of algebraic manipulations of the Gompertz equation.

The plot of text-figure 4 illustrates the general properties of tumor growth: 1) The specific growth rate shows a clear decrease with time. 2) The decrease appears to be linear on this semilog plot. If this is true, then the decrease observed is exponential with time. 3) The range of decrease observed in a single set of data is usually about 10- to 20-fold (in text-fig. 4 the decrease in specific growth rate is 20-fold; the range of tumor growth measured was about 200-fold). 4) This set of data passed through the inflection point of the Gompertz curve at 12.1 days. But there is no change in the specific growth rate at this point, no shift to another decay process. The inflection point is merely the point at which the numerical value of the specific growth rate happens to equal the numerical value of its rate of exponential decay (slope of the line in text-fig. 4). 5) The semilog line theoretically extends indefinitely in both directions; therefore, there is no numerical restriction on either the specific growth rate or its rate of decay.

I place so much stress on this explanation of the nature of the Gompertz equation, and with it the mathematical nature of the tumor growth curve, because there is evidence in the literature of a wide misunderstanding of precisely these facts. For example, the following statements have appeared in the literature: ". . . tumors had not yet reached the size when their exponential growth begins to decelerate according to the Gompertzian model" (*4*); necrosis was said to accelerate as the tumor passed through the inflection point ". . . when there was a change from mainly simple exponential to a more rapidly decreasing rate of growth" (*5*); ". . . for small tumors growth is nearly exponential" (*6*).



Text-figure 5.—Growth of the same tumor as shown in text-figure 4, before the inflection point. The Gompertz curve is the least-squares fit to the whole set of data. The exponential curve is calculated as $W = W_o e^{0.788t}$ to correspond with the Gompertz curve

$$W = W_o e^{\frac{0.788}{0.142}(1 - e^{-0.142t})}$$

Careful consideration of text-figures 1 and 4 should help to dispel this type of confusion: 1) Retardation, if it exists for a particular tumor (α significantly greater than zero), exists at all times for that tumor and continues at an unchanging rate throughout growth. 2) The size of tumor (W in equation 2) has nothing to do with the rate of retardation; some physically large tumors have little retardation [the W256 data of Schrek (*7*, *8*), for example (*1*, *9*)], whereas some tumors pass through the inflection point and approach a final limiting size while still physically quite small [mouse tumors often do this (*1*, *9*)]. The only factors that bear on whether growth of a tumor is nearer to or farther away from being simple exponential growth (and then *throughout* growth) is the ratio of the specific growth rate (A) to its rate of decay (α). If this ratio is high during growth, the tumor will follow a curve more nearly exponential, but still departing from it to simple visual inspection, as shown in text-figure 5. Text-figure 5 shows the preinflection measurements of the same tumor as shown in text-figure 4. The Gompertz curve fitted to the data is compared with the curve of simple exponential growth whose constant rate of increase equals the initial specific growth rate (as if α were zero). It is easy to see that both the data and the Gompertz curve are falling progressively behind a simple exponential well before the inflection point.

CELL KINETICS OF GROWTH RETARDATION

If retardation is a general law of growth, as it appears to be, then the question arises: What is happening to the cells of a tumor that this kind of growth should be observed? Both tumors and fetuses grow by cell division; if this is a steady, unremitting process, why is overall growth not exponential?

Two general possibilities come to mind. Either the average generation times are increasing as growth continues, or there is a loss of those cells whose multiplication causes the growth in the first place. When we first described the Gompertzian retardation of growth, we favored the former explanation because, if the latter were the principal cause, the cell loss would have to be increasing exponentially, and one would expect to identify the rapidly increasing number of dead cells rather easily. In fact, both Klein and Révész (*10*), studying the Ehrlich and MS1M ascites tumors, and Patt and Blackford (*11*), studying the Krebs ascites tumor, showed by vital staining procedures that the number of dead cells in these tumors was very small (1–2%) and remained small throughout growth. All three of these tumors are well fitted by the Gompertz equation (*1*, *9*). We must emphasize that the mechanism responsible for the growth retardation must be one that provides for a rapidly *increasing* loss of generative activity. A constant proportion of cell loss, for example, would merely result in a decrease in the constant rate of exponential growth; it would not change the growth from exponential to a retarded Gompertzian form, with its continuously decreasing rate of exponential growth.

In the past year, several studies have shown what appears to be sufficient cell death to account for the mathematically defined growth retardation of tumors. Autoradiographic methods were used in all these studies, which present a remarkably uniform picture of what happens in tumors at the level of cell replication.

In the first of these studies (*12*), in which a transplantable fibrosarcoma of C3H mice was grown as a solid tumor, an ascites tumor, and in tissue culture, it was shown that the tumor grew according to the Gompertzian model in all three growth forms. The duration of the cell cycle was the same *in vivo* and *in vitro*, and it did not change as overall tumor growth was undergoing the Gompertzian retardation. Growth retardation was shown to be due mainly to a decrease in the growth fraction and to a large amount of cell death, both *in vivo* and *in vitro*. Cell death was determined as the deficit between the relative birth rates of cells as estimated by autoradiographic means and relative birth rates as estimated by the measured increase in the number of tumor cells. These authors concluded that a "nonnegligible proportion of cells disappear immediately after mitosis—either dying or migrating from the tumor."

In a study of a transplanted C3H mouse mammary tumor (*13*), the doubling time of the tumor was compared when determined by calculation from autoradiographic parameters and by computer estimates from the overall growth measurements; the results suggested rates of cell death as high as 80%.

The studies mentioned above deal with animal tumors, and transplanted tumors at that. What about human tumors, which are of course spontaneous? Is retarded growth, accompanied by a very high incidence of cell death, characteristic of human tumors as well, or are we seeing a laboratory phenomenon with no bearing on cancer as a clinical entity?

This question was answered by Steel (*14*) in a review of the pertinent literature on autoradiographic data obtained on human tumors. Using the "thymidine-labeling index," he developed an equation that permits calculation of a "no-loss" doubling time of the tumor cell population. Then he compared this figure with the actual doubling times that have been recorded for human tumors, assuming cell loss to be the only cause of the discrepancy. The figures for actual doubling times are, of course, very crude; for one thing, few human tumors are measured for a sufficient time to yield a growth curve from which corresponding doubling times can be calculated. Nevertheless, the discrepancies were so large that there is no difficulty reaching a meaningful conclusion: The median cell loss in these human tumors was about 77%, and in general most human tumors probably have a cell loss greater than 50%.

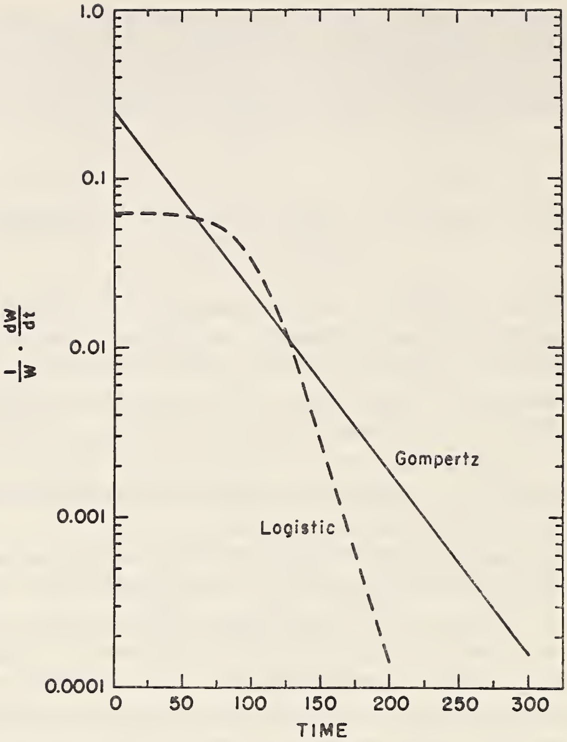
On the basis of these studies, the currently accepted view is that cell death is the major cause of the continuous deceleration of tumor growth defined and first demonstrated by the Gompertz equation. However, it should be mentioned for the sake of completeness that in another autoradiographic study aimed at identifying the mechanism of tumor growth retardation, Lala and Patt (*15*) failed to detect any cell death. They believed the cause to be an increase in the generation times.

PHYSIOLOGICAL MECHANISMS OF GROWTH RETARDATION

Not only at the cell level but at the tissue and/or organ level one must search for an explanation of growth retardation. Along this line, two recent studies have suggested that the growth retardation of tumors is merely what one would expect from deficiencies and ultimate failure in the supply of O_2 and nutrients to the tumor tissue (*6*, *16*). However, I do not favor this explanation, and, in its place, I will suggest a mechanism that would serve both normal and malignant growth equally well, as I believe any successful explanation must do.

First, the arguments against deficient blood supply as an explanation are the following: 1) The geometry of vascularization varies in such different tumor types as solid carcinoma, ascites tumors, and solid sarcomas, the last-named having characteristically a rich interstitial blood supply. Indeed, if poor vascularization with concomitant deficiencies in O_2 and nutrient supply were the cause of growth retardation, one would anticipate that sarcomas might grow by simple exponential growth, as was suggested in one of these studies (*16*). But no differences in the dynamics of growth have been observed among all the tumors so far studied, which include sarcomas and carcinomas, solid and ascites tumors, and primary and transplanted tumors. 2) The same growth retardation is observed in normal growth as in tumor growth (*1–3*, *9*, *17–20*), but we would not be tempted to conclude that a mouse would grow exponentially if it could only get enough air to breathe and food to eat. 3) Before a practical deficiency in O_2 and/or nutrient occurred, one would expect that growth would be essentially independent of the concentration of these materials, and then that growth would slow rather quickly as limitations became threatening to cell survival. This is in fact the kind of growth one sees in bacterial cultures, and such growth is well approximated by a logistic equation, whose derivative differs considerably from that of a Gompertz (text-fig. 6). 4) Noxious agents such as X irradiation do affect growth rate, but they do so by changing the parameters of the Gompertz equation, not by inducing Gompertzian growth where only exponential growth existed before (*21*, *22*).

As an alternative explanation for Gompertzian growth retardation and for the great cell loss recently demonstrated in tumors, let me suggest that growth retardation is a metazoan characteristic of organisms and their parts, including tumors, and that tissues retain this basic, genetically



Text-figure 6.—Semilog plot of the specific growth rate $\left(\frac{1}{W} \cdot \frac{dW}{dt}\right)$ of the Gompertz and logistic functions that were fitted by the method of least-squares to the original data on the prenatal and postnatal growth of the guinea pig—the same data as used in the preparation of text-figure 1.

programmed process when they become malignant. We know the majority of tumors retain enough of the normal morphogenetic mechanisms that they can be recognized readily by the pathologist as to their tissue of origin. If most tumors then retain this much differentiation, I suggest that growth retardation is likewise an expression of the retention by neoplastic tissue of some of the properties of the normal tissue of origin.

In support of this interpretation, I shall describe briefly the evidence for programmed massive necrosis as a mechanism of normal differentiation. A number of years ago, Glücksmann emphasized that cell death is one of the important morphogenetic processes in embryogenesis, along with cell multiplication and cell migration (*23*). He later showed that cell death is widely found, as a mechanism in invagination, evagination, foldings, formation of tubules, etc. (*24*).

More recently, sites of necrosis in embryonic development have been studied by the classical methods of transplantation used to define the fate of other presumptive areas in embryogenesis (*25*, *26*). In the avian embryo, for example, massive areas of necrosis appear in the development of the main joints. When such an area is removed from the posterior margin of the wing bud in the chick embryo, at stage 17 when it can be indentified, no necrosis develops at that site at stage 22 when it would otherwise appear. Moreover, the removed tissue, when transplanted to the dorsum of the embryo or to tissue culture, will proceed to necrosis at the normal time, duplicating the normally occurring necrosis even to the number of macrophages present (*26*). Determination of the area of necrosis is not irreversible, however, until only a short time before it occurs; central mesoderm of the wing or leg will prevent necrosis indefinitely, exerting this effect even through a fine filter.

I should like to suggest, therefore, that this preprogrammed cell death is a universal characteristic of metazoan growth, retained in tumors along with other features of normal growth and differentiation. In this case cell multiplication and cell death would both be preprogrammed, under the control of genetic mechanisms, and in tumors the two processes of cell production and cell loss are balanced at a point that yields the growth of new tissue.

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Cell Population Kinetics and Chemotherapy

G. G. Steel *and* L. F. Lamerton, *Biophysics Department, Institute of Cancer Research: Surrey, Belmont, Sutton, Surrey, England*

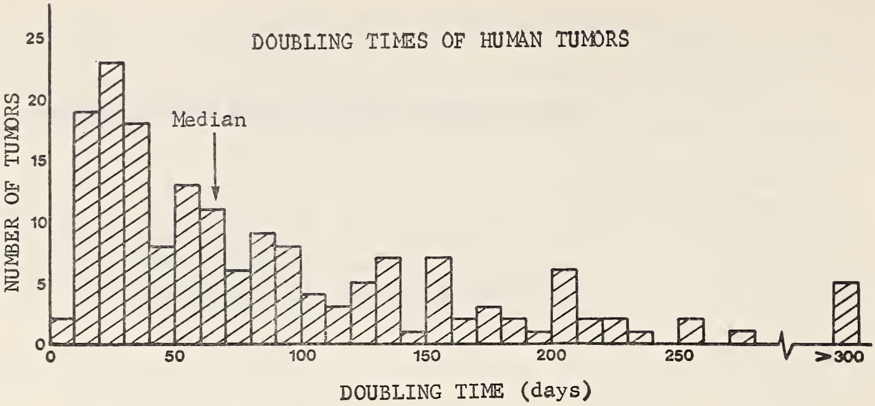
Part I: The Kinetics of Tumor Cell Populations (G. G. Steel)

THE work I am going to describe has been performed in the belief that what is mainly needed in the development of cancer therapy at the present time is a better understanding of the biology of tumors, particularly the kinetics of their growth. Our work has been much influenced by the recognition that human tumors grow very slowly; *i.e.*, the volume doubling times common for human tumors are much longer than the cell cycle times that many mammalian tumor cells have. Until we have a good idea of how human tumors grow slowly, we cannot understand their response to therapy.

Text-figure 1 illustrates the range of the available data on the volume doubling time of human tumors. It probably includes most of the published measurements of human tumor growth rate, covering a total of 175 cases studied by various authors. For the most part these were secondary tumors in the lung, but I have included also cases of primary tumors in various sites (*1*, *2*). There are a few tumors that have a volume doubling time of a week or less; a few with a volume doubling time of a year or more; and between these two limits there is a wide range with a median in the region of 60 or 70 days.

A tumor can grow slowly in three general ways:

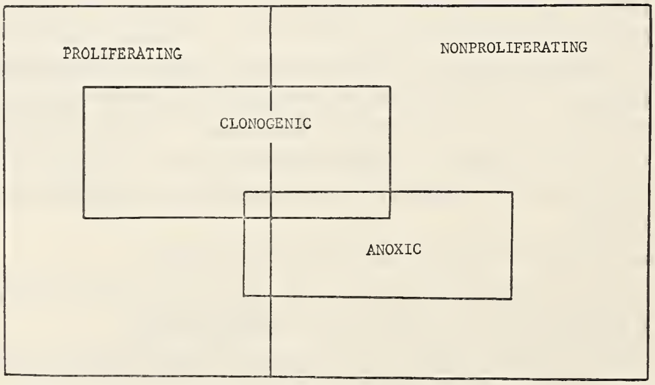
a) with long cell cycle times (cycle times comparable with the volume doubling time, and most cells proliferating);
b) with relatively short cycle times, but many cells not proliferating (a low "growth fraction");
c) with a relatively high rate of cell production, but extensive cell loss.



TEXT-FIGURE 1.—The distribution of 175 published measurements of the volume doubling time of human tumors (*2*)

If we are to predict how a tumor will respond to therapy, we at least need to know which of these broad categories it falls into.

At the present time, any discussion of tumor-cell population kinetics must greatly simplify the true nature of the cell population concerned. Recently Mendelsohn (*3*) well illustrated some aspects of the complexity of the problem facing us. Text-figure 2, one of the diagrams he used (*3*), shows some of the compartments into which a tumor cell population can be divided. It can be divided, for instance, into the cells which are proliferating and those which are nonproliferating, thus specifying the growth fraction. Within these compartments a certain proportion will be clonogenic, *i.e.*, cells capable of indefinite proliferation, and also there will probably be some cells which are in resistant states. With radiotherapy we think particularly of the resistance associated with anoxia but with chemotherapy there may well be other types of resistant state. Therefore,



TEXT-FIGURE 2.—Some possible subdivisions of a tumor cell population.

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within the concept of growth fraction, which is what we often measure, there are, from the point of view of therapy, a number of other extremely important factors. The two which I would principally emphasize are:

1) the proportion of clonogenic cells which have an enhanced resistance to cytotoxic agents;
2) the rate at which these cells can come back into a proliferative and sensitive state after the start of therapy.

Our ignorance of these parameters as regards chemotherapy is almost complete.

ANALYSIS OF TUMOR CELL KINETICS

I should at the outset disclose the fact that I am a convinced model builder. Some people scorn the use of theoretical models and regard this rather as a game for children. However, I regard the use of theoretical models in this field as the essence of the scientific method. I believe that we have to seek a theoretical model simulating as closely as possible the cell population of a tumor. Bearing in mind the restriction of Occam's razor, we must always be seeking the simplest model. Our ability to find such a model will indicate the extent of our knowledge about the cell population. And for the practical purpose of predicting the response to treatment, we need a model that will simulate the tumor with just sufficient precision to allow us to make a valid choice between therapies.

I am not going to go into any great detail about the theoretical form of the models we have used; I should just indicate that we think always in terms of a distribution of cell cycle times, and we seek information about this by use of the labeled mitoses technique. The basis of this technique is that first one gives a flash label of tritiated thymidine, thereby labeling the cells in the DNA synthesis phase at one particular time, and then one looks at the passage of the cohort of labeled cells through successive mitoses. The proportion of mitoses labeled shows a series of waves and the period between the peaks is a measure of cell cycle time. We analyze this type of data by use of the method developed by a mathematician (Dr. J. C. Barrett) in our laboratory. If all cells had identical phasing of the cycle, then each of the peaks of the labeled mitoses curve would come up to 100%. When there is a spread of cell cycle time, the initial synchrony will be gradually lost and the curve of percent labeled mitoses will damp out. In principle we can work back from the degree of damping to find what spread in cell cycle time gave rise to it. We use a computer for this, to synthesize theoretical labeled mitoses curves for specified values of the mean and standard deviation of the residence times in G_1, S, and G_2. We thus can deduce what distribution of cell cycle times is consistent with any particular set of experimental data.

Since the labeled mitoses technique is performed purely by looking at mitotic figures, it gives information only about the proliferating cells within the tumor cell population. To determine growth fraction we use a continuous-labeling experiment, recording the increase in the labeling index of all cells, both mitotic and interphase. The analysis of such data is not simple, since, during the course of continuous labeling, labeled nonproliferating cells will appear as descendants of labeled proliferating cells. Nevertheless, it is possible to ask whether a cycle time distribution, as found from the labeled mitoses analysis, is capable of explaining the shape of the continuous labeling curve and, if it is not, to find what proportion of nonproliferating cells is needed to give satisfactory agreement. This analysis thus sets up a model cell population in which there is a specified degree of spread in the kinetic parameters, and we try to find the form of such a model which will simulate the tumor with respect to the labeled mitoses and continuous labeling techniques (4).

The third parameter which I mentioned earlier is cell loss. Strangely, only in the last few years has the rate of cell loss from tumors been estimated, even though for some time the necessary data were available. The method we have developed for estimation of cell loss is, briefly, as follows. If we measure a thymidine-labeling index and the duration of the DNA synthesis phase, then the rate of production of cells within the tumor can be deduced. In doing this calculation, we have to correct for the nonrectangular age distribution of expanding cell populations (*5*). Having calculated a cell production rate, we can now find out the time it will take the cell population to double its size, assuming that no cells are lost. We have termed the doubling time in the absence of cell loss the "potential doubling time" of the tumor cell population. If we now compare this potential doubling time (T_p) with the true doubling time of the tumor cell population (T_d), we can deduce the extent of cell loss. We express the result as the "cell loss factor" (ϕ)

$$\phi = 1 - \frac{T_p}{T_d}$$

This factor indicates the rate of loss of cells as a fraction of the rate at which cells are being added to the population by mitosis. Thus a cell loss factor of unity (or 100%) indicates that the cell loss just balances cell production and there is therefore no overall growth or regression.

In general we do not know the true cell population doubling time. We usually know only the volume doubling time, and many factors can produce a discrepancy between these two parameters. We have examined the effect of this uncertainty and found that in most cases the use of a volume doubling time will underestimate the extent of cell loss (*5*). For instance, if the tumor is getting progressively more necrotic, then the cell population doubling time will be longer than the volume doubling time and the calculated value of ϕ is too low.

An example of the calculation of cell loss by this method is provided in the data which Mendelsohn (*6*) and Bresciani (*7*) have obtained on the growth of C3H tumors in mice. Mendelsohn found that the average volume doubling time for this tumor was around 8½ days, whereas the autoradiographic data of both of these authors indicate a potential doubling time of 2½ days. If these values are put into the expression for the cell loss factor, the result is a value of 70%, indicating that cell loss may be a relatively important factor in the growth rate of these tumors.

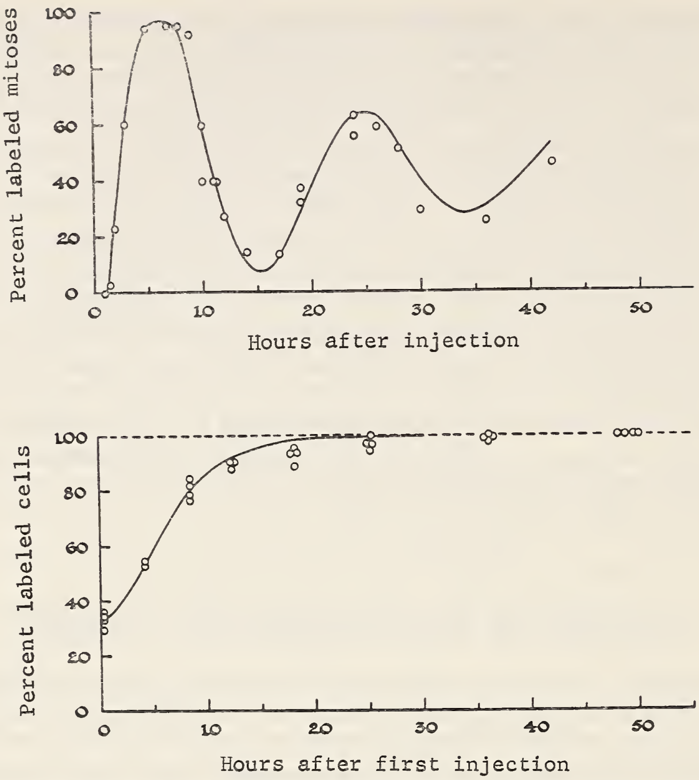
I should emphasize that the estimates of cell loss factor obtained by this method are not very precise—partly because the experimental data for labeling index, DNA synthesis time, and doubling time are often not very precise and also partly because the use of a volume rather than a cell population doubling time introduces considerable uncertainty. These estimates can, however, allow us to divide tumors broadly into those which seem to have little, moderate, or extensive cell loss, and, for the practical purpose of understanding response to therapy, it may well be that this is all we require.

KINETICS OF TRANSPLANTED RAT TUMORS

I would like now to talk briefly about the work we have been doing on three classes of tumor. In planning this work we felt that what matters is not how much we know about a particular type of tumor but how closely it resembles the common forms of human cancer. One major problem at the present time is to find experimental tumor material which is as "realistic" as possible but which still allows us scope for experiment.

First I want to describe the work we have been doing on transplanted rat tumors. Obviously, transplanted rat tumors cover a wide range of tumor types, and I feel that at the present time our understanding of their growth kinetics is poor. Much more data are necessary before we can begin to correlate cell population kinetics with such factors as growth rate, number of passages, and tissue of origin. Most available data are on rapidly growing, well-established transplanted tumors, an example of which is the BICR/M1 tumor (text-fig. 3). This tumor had a volume doubling time of just less than a day. Without detailed analysis, it can be seen that the average cycle time was close to the volume doubling time and that, under continuous labeling for 24 hours, over 95% of the tumor cells became labeled. Therefore, almost all the cells were proliferating rapidly and there was little cell loss.

In more slowly growing tumors the situation is different. Text-figure 4 shows the results on a tumor (BICR/A2) which was in only its fourth transplant generation at the time of the experiment and which had a volume doubling time of about 8 days. The labeled mitoses analysis indicates a mean cell cycle time of about 60 hours, with a rather large standard deviation. In the continuous labeling experiment, only 70% of the cell



Text-figure 3.—Labeled mitoses and continuous labeling curves for a rapidly growing, transplanted rat tumor (BICR/M1) (*4*).

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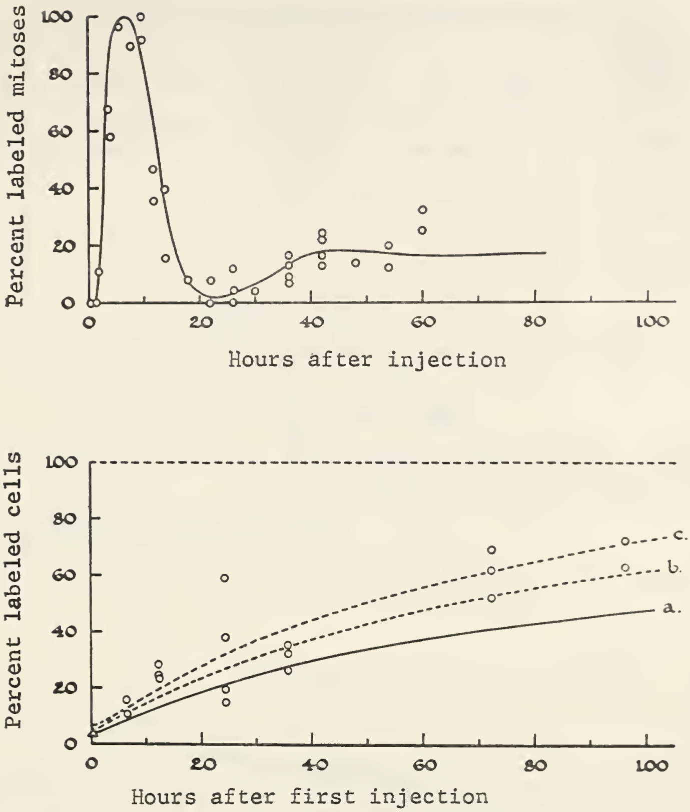
population became labeled in 4 days. One cannot read off the growth fraction directly from a curve of this type, but our analysis indicated that the data were consistent with a growth fraction of 30% and some degree of cell loss from the nongrowing compartment.

The distributions of cell cycle time obtained by the Barrett method for the tumors BICR/M1 and BICR/A2 are compared in text-figure 5.

There are many reasons for dissatisfaction with well-established transplanted tumors in the extent to which they resemble human cancer. One of our main objectives at the moment is to see whether, with early transplants of spontaneous and induced rat tumors, we can obtain more "realistic" tumor systems. Rather surprisingly to me, one can find some extremely slowly growing tumors in rats. Text-figure 6 shows an example of the type of transplantable tumor which we are at present seeking. This was a spontaneous mammary tumor in a female August rat, of which the primary tumor had a volume doubling time of about 60 days. On transplantation into recipients of the same strain and sex, all 5 tumors grew with good uniformity, the average volume doubling time being about 40 days.



TEXT-FIGURE 4.—Labeled mitoses and continuous labeling curves for a transplanted rat tumor (BICR/A2), whose volume doubling time was about 8 days. *Broken lines* indicate the effect of assuming that nonproliferating cells have a limited lifespan of 1 or 1.5 times the volume doubling time (curves *b* and *c*, respectively) (*4*).

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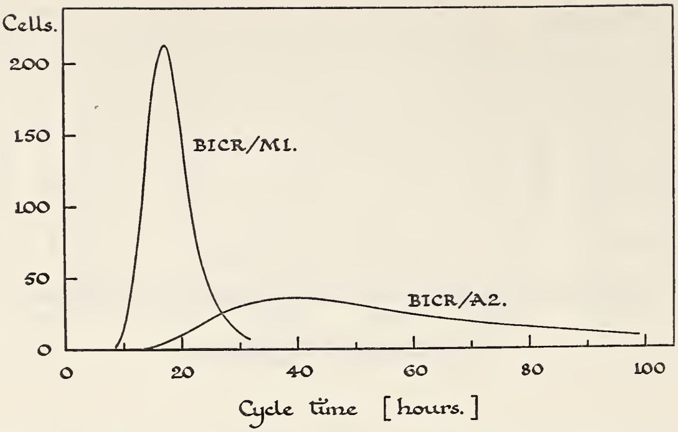
Growth was arrested by oophorectomy and was stimulated somewhat by implantation of pellets of estradiol. We feel that this type of tumor gives us the opportunity to investigate the growth kinetics of tumors whose volume doubling times are well into the range found for human tumors (text-fig. 1) and also to study their response to various types of treatment.

At the present time this program is in its early stages, but we do have information on cell loss from the primary tumors. Since we do not have individual measurements of the duration of the S phase in these tumors, I have presented the data in the form of a chart (text-fig. 7). On this type of plot, for any given duration of the S period, the no-loss condition gives a linear relation between labeling index and population doubling time. For these tumors it will be assumed that the volume doubling time indicates an approximate cell population doubling time, and throughout the present discussion the value of λ (*2*, *5*) will be taken as 0.75. I have chosen



Text-figure 5.—Distributions of cell cycle time which are consistent with labeled mitoses curves for tumors BICR/M1 and BICR/A2.

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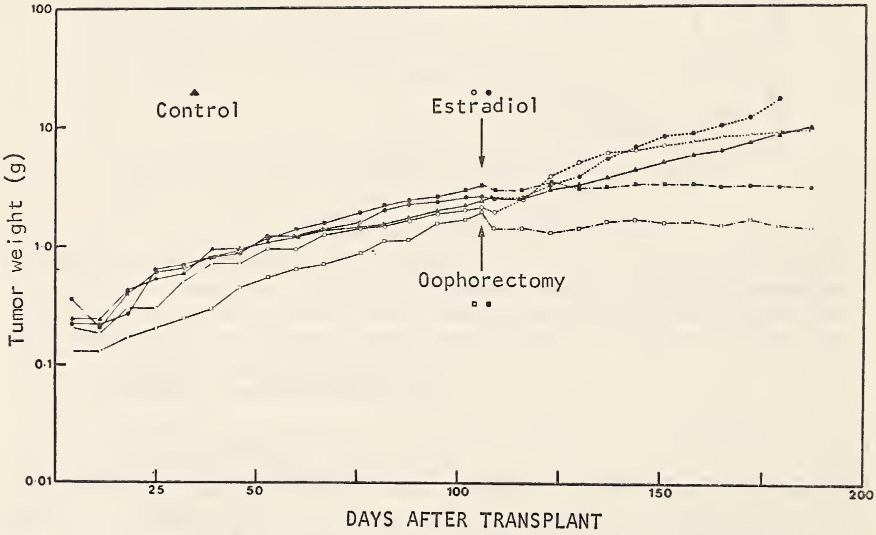
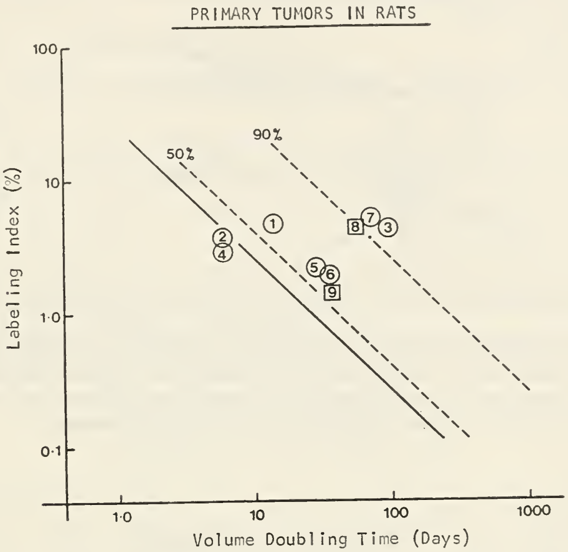


Text-figure 6.—Growth curves for 5 individual transplants from a spontaneous rat adenofibroma (BICR/A8). At 106 days after tumor transplantation, when the overall volume doubling time was about 45 days, 2 animals were oophorectomized and 2 were given 10 mg subcutaneous implants of fused estradiol.

8 hours for the duration of the S period and have indicated where points should fall for a cell loss factor of 50 or 90%. The data for the primary rat tumors show a broad range; in some cases the points fall close to the no-loss condition and in others there is evidence of up to 90% cell loss. The more slowly growing tumors in this series have volume doubling times of 30 days or more, and they seem to have the highest degree of cell loss. Al-

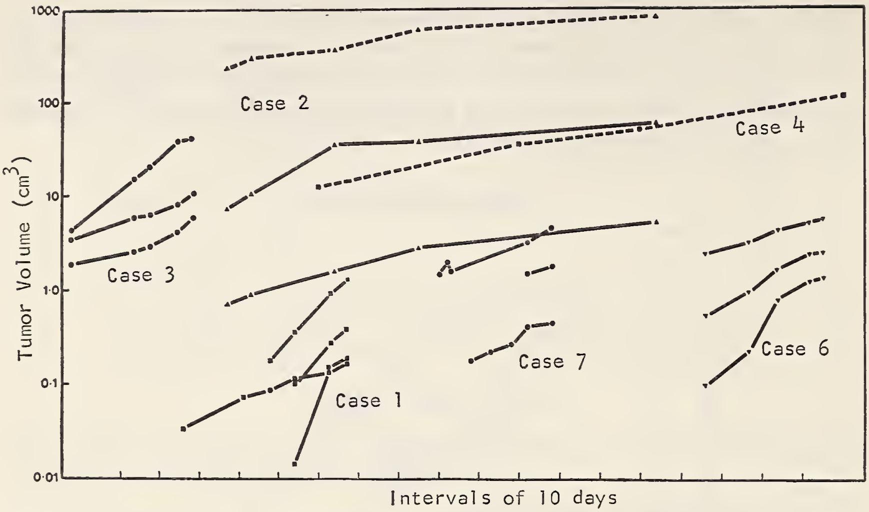
though we can find experimental tumors which are comparable in growth rate with many human tumors, we must not of course imagine that this is all that matters. Other factors, such as the degree of malignancy, are also important, and we are only at the beginning of the search for the best form of experimental tumor material.



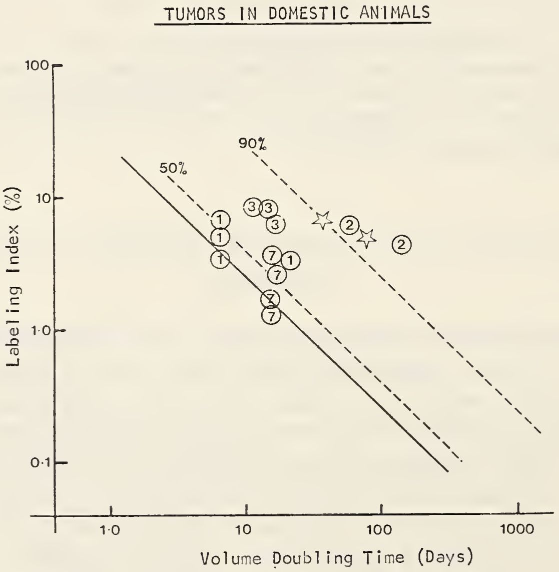
Text-figure 7.—Correlation between thymidine labeling index (1 hr after ^{3}H-thymidine) and volume doubling time in 9 primary rat mammary tumors. Those tumors represented by *circles* were induced by 400 rads whole-body radiation; those represented by *squares* arose spontaneously in breeding females. Theoretical lines for 0, 50%, and 90% cell loss are shown.

KINETICS OF SPONTANEOUS TUMORS IN DOMESTIC ANIMALS

The second line of investigation which I want to describe is our work on spontaneous lung metastases in dogs. This work is being done in collaboration with a veterinarian, Mr. L. N. Owen, of Cambridge University. We select animals with proved neoplastic disease, concentrating on those which have measurable lung metastases. We follow the growth of these tumors by serial chest radiographs and then give thymidine shortly before killing the animal. Text-figure 8 shows some growth curves. By measuring the terminal tumor growth rate, we can measure simultaneously volume doubling time and labeling index, which to my knowledge has not so far been possible in human cancer. Thus, from this material we can also gain evidence of the importance of cell loss (text-fig. 9). Once again I have taken a value of 8 hours for the duration of the S period. So far we have only been able to measure this in one primary



TEXT-FIGURE 8.—Growth curves for spontaneous tumors in domestic animals. All were dogs except case 4 which was a cat. *Solid lines* indicate the growth of lung metastases, determined radiographically. *Broken lines* show the growth of two primary tumors. The lateral position of the curves is arbitrary.



TEXT-FIGURE 9.—Correlation between thymidine labeling index (1 hr after ^{3}H-thymidine) and volume doubling time in spontaneous tumors of domestic animals. *Circles* enclosing the case number represent lung metastases; *stars* represent primary tumors.

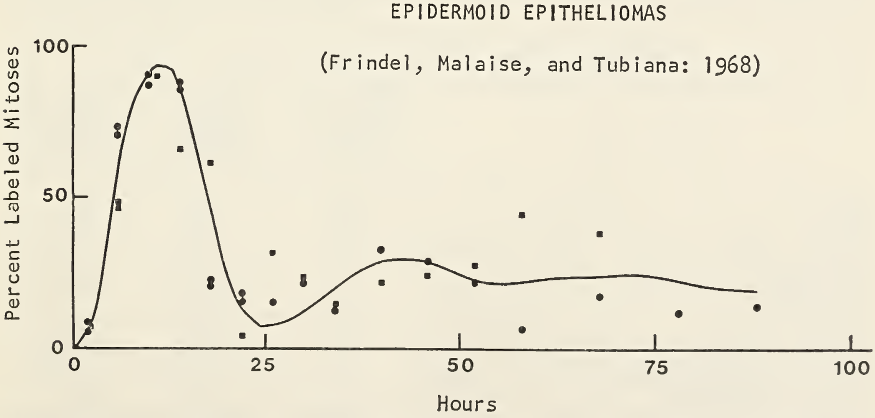
canine tumor and the result agreed well with measurements which have been made on transplanted rat tumors. The labeling index and growth rate data broadly resemble those on the rat tumors. In some cases there is little or no evidence of cell loss; in others cell loss may approach 90%. We have at present too little data from which to examine the correlation between cell loss factor and growth rate or histopathology.

CELL LOSS FROM HUMAN TUMORS

Finally, I want to discuss work which has been done on cancer in man. At the present time the labeled mitoses technique has been used only on a few human solid tumors, principally by Tubiana and his co-workers in Paris (*8*). Tannock, in our department, recently made a computer analysis of these data, and text-figure 10 shows the result for the two epidermoid epitheliomas, the cases on which the data are most complete. The computed curve corresponds to the following cell cycle parameters, measured in hours (mean ± standard deviation)

G_2 period: 6 ± 3 (median 5.4)
S period: 12 ± 2 (median 12)
G_1 period: 40 ± 60 (median 22)

The median duration of the whole cycle was 40 hours. The implication of this work is that the cycle times of cells within these tumors were considerably shorter than the probable volume doubling times. Most cells seem to have had cycle times of less than 2 days, and from the clinical history the volume doubling time of one of the tumors was about a month. From the initial labeling index, which was about 8% in these two cases, the authors estimated that the growth fraction was about 40% but it is likely that cell loss was also important.



TEXT-FIGURE 10.—Labeled mitoses curve for 2 human epidermoid epitheliomas, studied by Frindel *et al.* (*8*). *Solid line* has been computed for the cell cycle parameters given in the text.

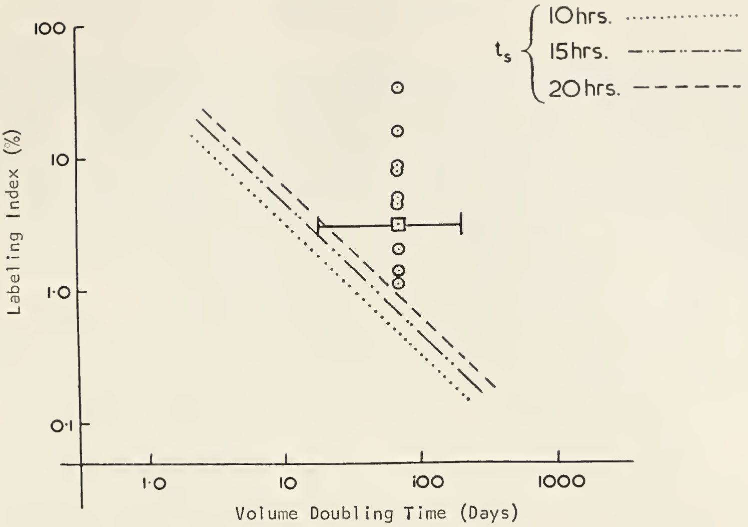
There is other evidence that cell loss may be an important factor in determining the growth rate of human tumors (*2*, *9*, *10*). The method I have outlined for calculating the cell loss factor requires data at least on thymidine labeling index and volume doubling time. So far as I know, these two parameters have never been measured simultaneously in the same patient but there is a good deal of data on each parameter separately. Thymidine labeling index has mainly been determined by *in vitro* methods, and in table 1 I have summarized much of the data in the literature [*see* (*2*) for sources of these data]. I have classified the results from 170 cases according to the tumor site; for each site I have calculated the median labeling index and also, by assuming an S period of 15 hours, a potential doubling time. Clearly, there is some uncertainty about the correct value to take for the S duration, but this is the mean for the 5 tumors studied by Frindel *et al.* (*8*). The overall median potential doubling time is 15.6 days, considerably shorter than the median of 66 days for the distribution of human volume doubling times (text-fig. 1). Details of the source and anatomical site of the tumors from which this distribution was made have been given in an earlier publication (*2*). It should be stressed that the types of tumor from which the presently available labeling index and doubling time have been obtained are rather different, the former being lung metastases and the latter mainly primary tumors; the comparison of these two groups of data must therefore be tentative, but the growth rate of the primary tumors will, if anything, be slower than that of the lung metastases. In text-figure 11 the human data are presented in the same type of plot that was used for the rat and canine tumors.

Within the limitations of this type of comparison, it can be seen that the bulk of the available human tumor data falls to the right-hand side of what could reasonably be taken as the appropriate no-loss condition.

TABLE 1.—Labeling indexes of human tumors

Site of tumor	Number of measurements	Median labeling index(%)	Potential doubling time* (days)
Breast	38	1. 1	43
Colon	31	4. 5	10. 4
Somach	11	6. 9	6. 8
Central nervous system	15	2. 0	23. 4
Melanoma	8	3. 3	14. 2
Lung and larynx	8	15. 0	3. 1
Tongue	6	7. 2	6. 5
Uterus and cervix	6	4. 8	9. 8
Lymphosarcoma, Burkitt tumor	9	32. 0	1. 5
Miscellaneous sites	38	1. 4	33
All sites	170	3. 0	15. 6

*Calculated for t_s = 15 hours.



Text-figure 11.—Correlation between thymidine labeling index and volume doubling time for human tumors. *Broken lines* indicate the no-loss conditions for 3 values of the duration of the S period (*2*). *Circles* indicate the median labeling indexes of each of the tumor site categories shown in table 1. *Horizontal bar* indicates 80% of the range of the volume doubling time data. *Square central point* indicates the median of both parameters.

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The cell loss factor computed for the overall median, taking the S period duration as 15 hours, is 77%.

A number of aspects of this type of calculation cannot be accepted without more detailed examination (*5*), but I feel justified in suggesting that the data available on human tumors imply that most of these tumors have a cell loss factor exceeding 50%. As we move away from the rapidly growing experimental tumors, on which most work has at present been done, there seems to be evidence for cell loss becoming of considerable importance in the growth rate of tumors.

I cannot here go into the details of the biological nature of cell loss from tumors. Many mechanisms may be responsible: exfoliation from carcinomas, spread of cells throughout the body, and cell death within the tumor. Cell death may well be the dominant mechanism. However, there are many types of cell death: the death of cells at the boundary of a necrotic region; the isolated death of cells in well-nourished regions (perhaps in abortive mitosis or as a result of an immunological challenge); and the "physiological" death of cells that have reached the end of their differentiated lifespan and die just as they would have done in the process of normal cell renewal.

CONCLUSIONS

I have emphasized in this presentation cell loss from tumors, because this is where much of our recent work has been concentrated and because the other kinetic studies are not yet complete. I do not claim that this is the first time that cell loss from tumors has been demonstrated; on the contrary, cell loss has always been regarded as a basic characteristic of malignant growth. What this work has shown is that we can now get reasonable, if rather approximate, estimates of the extent of cell loss, and that in many cases this appears to be extremely large.

The general implication is that we must regard many tumors not as simple expanding cell populations, whose growth can be stopped only by interference with cell production, but rather as renewal systems which are out of balance—systems in which the rate of cell production only slightly exceeds the rate of cell loss. In this situation the overall growth rate of the tumor, which clinically is what usually concerns us, is critically determined by the competition between cell production and cell loss. It may be that in the search for cytotoxic agents this competition has often been overlooked; many such agents have been selected because of their effectiveness against types of experimental tumors in which cell loss may be small. Where cure is the only objective, it may be right to attempt to kill all cells which have indefinite proliferative capacity. But in cases where we are willing to consider merely an improvement in prognosis, we should perhaps look more carefully at the competition between cell production and cell loss and seek ways of taking sides. I would join with those who at the present time emphasize the importance of control rather than obliteration as an approach to many types of human cancer.

Part II: Cell Population Kinetics After Therapy (L. F. Lamerton)

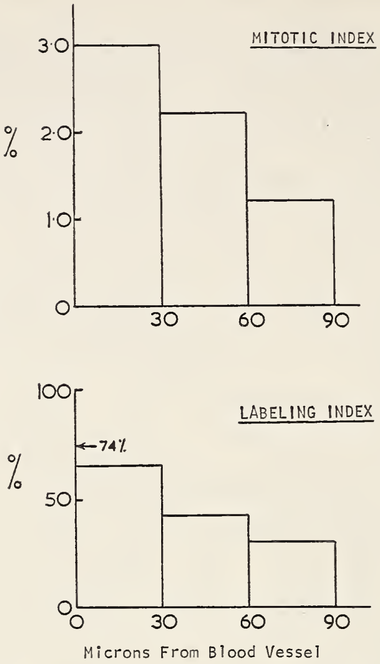
Dr. Steel has discussed the need for a deeper understanding of tumor biology as a requirement for the development of therapeutic methods, and has considered various aspects of the biology of the untreated tumor. I will discuss, in fairly general terms, aspects of the biology of the *treated* tumor and of the limiting normal tissues. To provide a proper basis for the scheduling of drugs in chemotherapy, we want to know how the response of the tumors and of the limiting normal tissues to the agents used will vary throughout treatment, and this must involve a knowledge of the changes that take place in the pattern of cell proliferation.

CHANGES IN PATTERN OF CELL PROLIFERATION IN TUMORS

Unfortunately very little work has been published on tumor response. There has been an increase in experimental studies of the change of anoxic fraction during radiation treatment, which may indeed have considerable therapeutic importance, but the amount of work on change in the cell proliferation pattern in tumors is still quite small. Using the labeled mitoses technique, Denenkamp and Fowler, at Hammersmith Hospital, measured the cell cycle of a series of solid tumors both before treatment and 1 or 2 weeks after a single dose of 1500 rads; they found essentially no change in cell cycle. My colleague, Bensted, studied some transplantable mammary tumors in the rat subjected to continuous irradiation at 176 rads per day; he found only slight changes in cell cycle. In one case he found a slight lengthening of the cell cycle and in another he found a slight shortening, but not a very marked effect. Some unpublished data of Van Peperzeel and Breuer in Amsterdam (*11*) suggested that the labeling index of the viable cell population in certain transplanted mouse tumors shortly after irradiation was considerably greater than that in the controls. With human material Tubiana's group in Paris determined labeled mitoses curves for an epithelial tumor, one half irradiated and the other half unirradiated; they obtained evidence suggesting that irradiation shortened cell cycle time somewhat. However, at the present time the data are not such that one can come to any firm conclusion concerning the way in which the pattern of cell proliferation in tumors will change during treatment. To follow-up this subject we have to think in more general terms, and ask what determines the cell cycle of tumors, which we know can vary over a very wide range. We have to ask how far the cell cycle time, or rather the distribution of cell cycle times, in a tumor is determined by the environment in which the tumor cell is growing and how far by its inherent characteristics. Here the work of Tannock (*12*) is of some relevance.

Tannock has been using a transplantable mouse tumor in which the viable tissue is found in cords around the blood vessels. Outside the viable tissue is a necrotic region, the distance from the blood vessel to the necrotic zone being about 90 μ. Tannock has studied the proliferation characteristics of the tumor in 3 zones—near the blood vessel, midway between the blood vessel and necrotic area, and near the necrotic area. Both mitotic and labeling indexes fall as one moves away from the blood vessel (text-fig. 12). On the other hand, labeled mitoses curves indicate that the cell cycle distribution is substantially the same in the 3 zones (text-fig. 13). Certain problems are encountered, since cells are migrating from the region of the blood vessel to the necrotic zone, but the analysis indicates that increase in distance from the blood vessel has little effect on cell cycle time, but does lead to a considerable reduction in growth fraction. In other words, if the cells are going to divide, they will do so at a standard rate, but the probability that they will embark on a new cycle is reduced as they move towards the necrotic zone. Calculations of diffusion distances suggest that increasing anoxia is the major factor in producing the change in cell proliferation pattern, but one cannot be certain that either a reduction in the availability of other metabolites or the diffusion of toxic products from the necrotic region does not also play a part.

HUMAN TUMOR CELL KINETICS

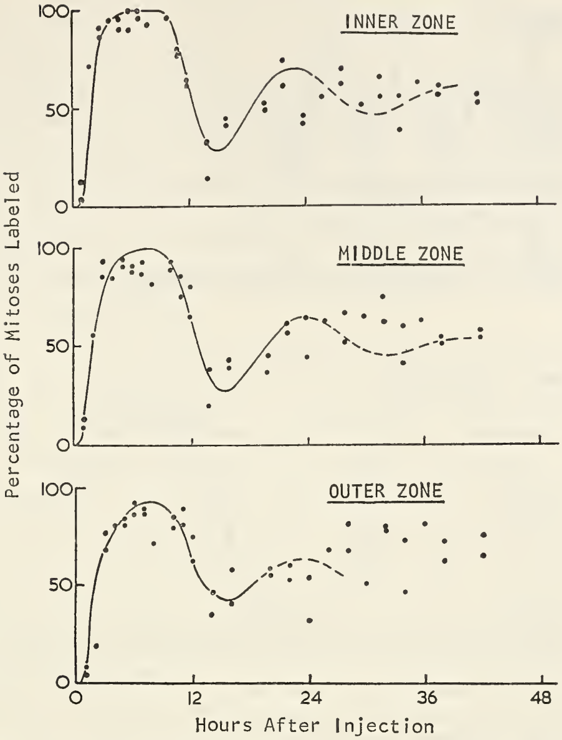


Text-figure 12.—Labeling and mitotic indexes in 3 zones of the cords of viable tissue within a transplanted mouse adenocarcinoma (BICR/SA1). The labeling index of cells that appeared to be in contact with the axial blood vessel was 74%.

A similar constancy of cell cycle in a tumor under changing conditions was shown by Frindel and Tubiana (*13*) in their studies of a mouse tumor at various times after transplantation.

However, with ascites tumors, the situation is very different. As has been demonstrated by Lala and Patt (*14*), by Frindel and Tubiana (*13*), and by Wiebel and Baserga (*15*), the cell cycle increases considerably with age. Labeled mitoses curves determined by Tannock for Ehrlich ascites tumors at different stages of growth are shown in text-figure 14, which illustrates very clearly the extent of the change in the cell cycle. When Tannock studied the same tumor grown in solid form, he found essentially no difference in cell cycle between 2 and 10 days after transplantation.

Why do solid tumor and the ascites tumor behave differently? There are profound differences in oxygenation, since at any given time most cells in the ascites tumor will be far from a blood vessel. Also in the ascites tumor there is likely to be an increasing concentration of toxic products with age, and immunological factors could well be playing a more important role than in solid tumors. On the other hand, the presence of



Text-figure 13.—Labeled mitoses curves for mitotic figures found within the 3 zones of the tumor cords in BICR/SA1. Theoretical curves were computed on the assumption of median cycle times of 16 hours for the inner zone and 17 hours in the middle and outer zones. Cell migration probably distorts the data beyond the second peak.

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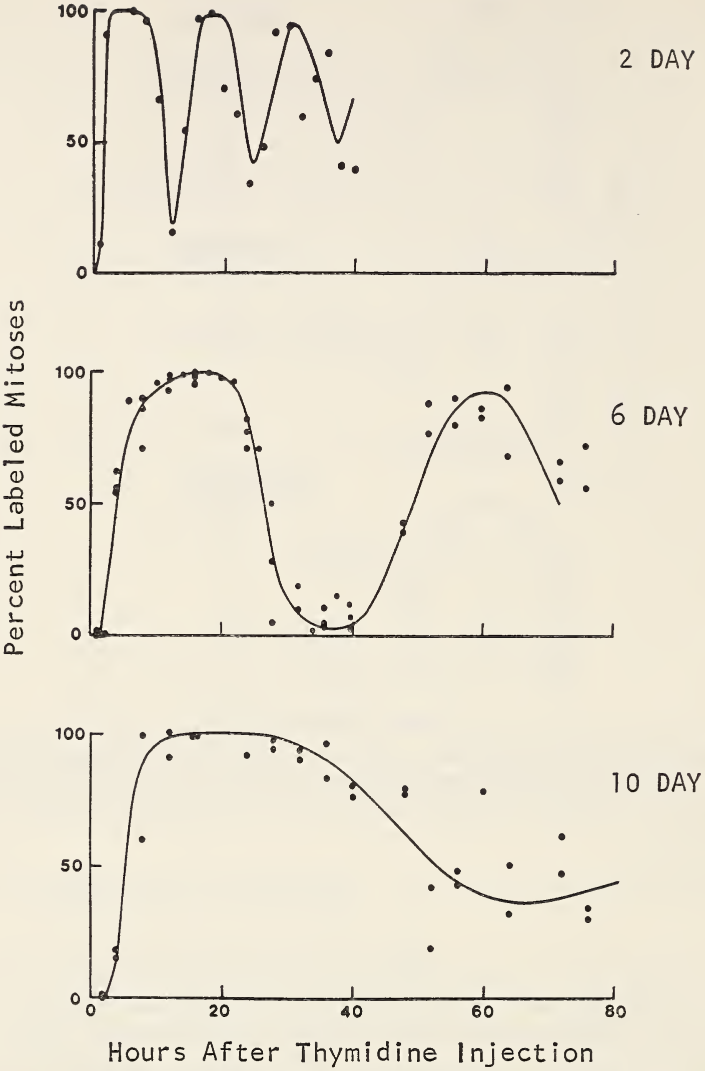
discrete necrotic zones in the solid tumors investigated could be the major factor, by causing cells to be absorbed before they had time to demonstrate any lengthening of the cell cycle. The current work of Malaise and Tubiana, comparing the response of solid, ascites, and cultured tumors to various factors, should provide some important clues.

A full understanding of the reasons for the difference in proliferative behavior of solid and ascites tumors is important, both practically and fundamentally, and one would also like to know how far human tumors resemble the experimental solid or the ascites tumors. This has a bearing on the possibility of substantial changes occurring in cell cycle time during treatment, as well as on the general problem of the value of ascites tumors as model systems for chemotherapy. As Dr. Steel indicated, we need to study the response of slowly growing animal tumors, which approximate as closely as possible the growth rate and other characteristics in human tumors.



TEXT-FIGURE 14.—Labeled mitoses curves in Ehrlich ascites tumor at 3 intervals following the implantation of 1.7×10^6 cells.

THE "G_0" CELL

Related to possible changes in the proliferative pattern of tumors are the problems surrounding the G_0 cell—the cell which is out of the division cycle but which can be recalled into division under the appropriate stimulus. There has been much theorizing on this subject, but very little in the way of direct experimental data. It is, I think, necessary to distinguish between the G_0 state as it exists in at least some normal tissues and the G_0 state as it may exist in tumors.

In certain normal tissues, such as the liver, a considerable part of the normal intermitotic time appears to be taken up by the time that passes while the cell is waiting to be triggered into preparation for division, the rate of release from the G_0 state changing with circumstances and representing one of the major control mechanisms of the rate of cell proliferation. Whether such a period of waiting is always part of the cell cycle in normal tissues, even in rapidly dividing tissues, is not known, but it is a reasonable hypothesis.

This concept of G_0 is different from that which has often been assumed to exist in tumors, where there is not a controlled release from a G_0 state but where certain cells, as a result of some environmental factor, can remain for a long time without dividing, but still retain proliferative potential.

What are the environmental factors that could keep a tumor cell in this state? Hypoxia is an obvious candidate for the role, particularly since in the corded tumor it seems to be reduced oxygen tension that decreases the growth fraction. We do not know how the capacity for recall into division depends on the degree and duration of the hypoxia—and this is an important field for experimental work—but if a degree of hypoxia, produced by poor vascularity, is a major factor in holding potentially proliferative cells out of cycle, the problem is a double one in the use of cytotoxic drugs. Not only are such cells likely to be resistant to the action of many drugs but the poor vascularity will make it difficult for the drug to reach them in sufficient concentration. In addition, there may be an "oxygen effect" in response, as with radiation, but insufficient work has been done to allow a conclusion to be reached on this point.

Among the various uncertainties, one which has not received a great deal of attention is how long a cell in a growing tumor might be expected to remain in a state of suspended proliferation. If the environmental factor concerned is hypoxia, it is unlikely that the cell could remain for long without dying, or without being reoxygenated, particularly in a treated tumor. The same reasoning could apply to other nutritional deficiencies, and the assumption that tumor cells can spend long periods in a G_0 state may well be justified. Theory has gone as far as it can on this question of the G_0 cell in tumors, and we need a great deal more experimental work on the difficult problem of distinguishing G_0 cells from sterile cells or those which may be proceeding slowly through the cycle.

The assumption that clinically one is dealing with tumors whose rate of cell division is greater than that of the vital normal tissues of the body is the basis of much of the experimental chemotherapy work that has been done. For some types of malignancy it may be true, but for most solid tumors it is probably not, and the cycle times of cells in the tumor are likely to be longer than those of cells in the gut and possibly the bone marrow.

HUMAN TUMOR CELL KINETICS

LIMITING NORMAL TISSUES

Before the general problem of the limiting normal tissues in chemotherapy is considered, it will be useful to digress for a moment and consider the problem in radiotherapy, since it has a bearing on the possible combination of chemotherapy and radiotherapy. Surprisingly little experimental work has been done on the effects on normal tissues that limit treatment in radiotherapy. We radiobiologists have tended to concentrate our efforts too much on the tumor itself and have not always recognized that the success of any cancer therapy must depend on achieving a sufficient differential effect between malignant and normal tissues. The importance of this is very evident in the present stage of radiotherapy, where the radiation doses given are, on the whole, very close to the tolerance of the surrounding normal tissues. At a recent meeting, a number of radiotherapists gave examples of how relatively small changes in treatment pattern could lead to an unacceptable increase in late effects on normal tissues—*e.g.*, changes from 4 to 3 fractions in a week, or increases in dose by less than 10%. It was the general view that an improvement of as little as 10% in the differential response of malignant to relevant normal tissue could improve appreciably radiotherapy results.

More biological work has not been done on the late effects of radiotherapy on normal tissues partly because these effects have not been precisely defined, and partly because the tissues concerned are not very amenable to present techniques of cell biology. Fibrotic conditions are a major factor in the late effects, and direct radiation damage to the fine vasculature probably plays a part, but the precise type of damage and the contribution of other factors are still largely a matter of opinion.

The relevance of this to chemotherapy is that, if only about 10% improvement in differential is required, this might be obtained by a combination of radiotherapy and chemotherapy. The important question is whether the effects in the immediate neighborhood of the tumor are different from those developing after localized radiotherapy. If a given drug can kill some tumor cells without increasing the severity of the limiting late effects from the radiation treatment, combined treatment could permit the necessary improvement in efficiency, even though the drug might not have a highly localized concentration in the tumor. I know of no experimental work in this field. Some clinical data, for instance, those of Fletcher and Suit using a combination of radiation and nitrogen mustard, suggest no therapeutic advantage. On the other hand, there is evidence, such as that from our own hospital with regional perfusion techniques, that methotrexate does not affect the dose of radiation which can be given subsequently.

With chemotherapy alone, the limiting normal tissues do, of course, vary with the drug used, but gut and bone marrow are often predominant by virtue of their high rate of cell proliferation and vital function. With radiation the differential between malignant and normal tissue is improved by protraction or fractionation. However, one cannot assume that this sparing effect of fractionation will necessarily apply to drugs, at least to those whose action depends greatly on rate of cell proliferation.

Our own radiobiological studies of the response of the small intestine and bone marrow of the rat show that both these tissues can tolerate large doses of radiation if the treatment is protracted (*16*). However, the state of each of these tissues under continuous irradiation is characterized by a more rapid rate of proliferation of the stem cells. In the small intestine the cells at the base of the crypts speed up their rate of division substantially. In the bone marrow the stem cells of the red cell series (measured by "repopulating ability") appear to decrease their turnover time from about 30 to 12 hours under continuous radiation at 45 rads per day (*17*).

Such changes in proliferation rate apparently do not affect very substantially the response to radiation, but they will increase response to certain drugs. The greater sensitivity of regenerating tissue could be a serious problem in chemotherapy. Recognizing this, Bruce and Meeker (*18*) have warned against treating regenerating bone marrow with drugs.

There is certainly a field for experimental work here, to study the way in which cell proliferation pattern (including cell synchronization) and cell sensitivity will change during drug treatment in bone marrow and gut. The work of Frindel *et al.* (*19*) indicated very interesting possibilities of synchrony in bone marrow cells following irradiation. Such studies may give some pointers for more appropriate scheduling of drugs in clinical chemotherapy. But one must not ignore the problem of extrapolation from the experimental animal to the patient, where the pattern of cell proliferation in both malignant and normal tissue may be very different from that in the animal.

Another line of work which may prove fruitful is the study of the synergism or antisynergism of various drugs and radiation, with relation to the changes produced in the cell proliferation pattern of both normal and malignant tissues. One may cite the work of Smith and her colleagues, on the way in which radiation damage to the bone marrow is effectively reduced by pretreatment with endotoxin or colchicine (*20*, *21*).

The subject of cell population kinetics is not yet at a stage where it can offer much in the way of suggestions for improvement in clinical chemotherapy, but we are all beginning to realize that a full understanding of the biology of our test systems in relation to the clinical conditions requiring treatment is an essential part of any program of expermental chemotherapy.

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Kinetics of Granulocytopoiesis[1]

EUGENE P. CRONKITE, *M.D.*,
Medical Research Center,
Brookhaven National Laboratory,
Upton, New York 11937

PEOPLE have been interested in the kinetics of granulocytopoiesis for many years, and there has been a whole spectrum of approaches to its study, perhaps the earliest ones being the use of benzol and whole-body irradiation to deplete the animals and study the rates of depletion and repletion (*1*). Tissue culture and ^{32}P were used by Osgood (*2–4*), one of the pioneers in the study of granulocyte kinetics; the mitotic index was used by Killmann and others (*5–9*); cinemicroscopy by Boll (*10*); colchicine arrest of mitosis by Dustin (*11*); sodium phosphate labeling of cells followed by transfusion and cross-transfusion in animals by Van Dyke and Huff (*12*) and Hollingsworth *et al.* (*13*); the radioactive phosphorus specific activity in DNA of separated cells by Ottesen, Osgood, and co-workers (*14*, *15*); the transfusion of Pelger-Huet cells by Rosse and Gurney (*16*); labeling with radioactive phosphorus during leukophoresis by Craddock *et al.* (*17*) and Bierman *et al.* (*18*). More recently another series of studies has yielded more information on the finer structure of granulocytopoiesis, namely the use of diisopropylfluorophosphate (^{32}DFP) for labeling granulocytes *in vitro* and *in vivo* and thereafter following their behavior. The use of tritiated DFP, measurements of the specific activity, and also, to a limited extent, autoradiographic studies by Wintrobe's group (*19–22*) and Perry and his associates (*23*) have been productive.

Tritiated thymidine (^{3}H-TDR), a specific precursor of DNA, which is suitable for autoradiography, was in 1956 simultaneously synthesized in Belgium (*24*) and at Brookhaven National Laboratory (*25*) and has been used intensively since then by us (*26–33*), Patt *et al.* (*34–37*), and by other investigators throughout the world. ^{3}H-TDR has been used by Perry and associates (*3*), both by autoradiography and by measurement of the specific activity of the cells.

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[1] Research supported by the U.S. Atomic Energy Commission.

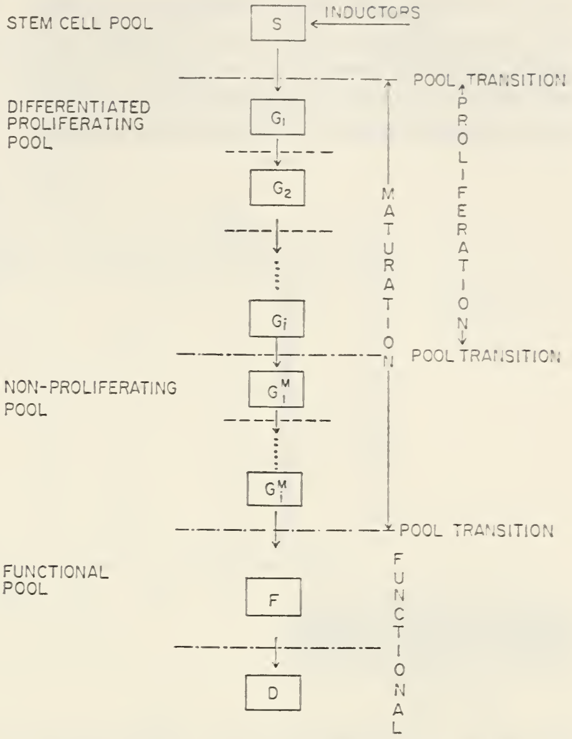
I will limit my comments exclusively to our own work, not from a sense of megalomania, but because I think you will agree that serious problems have arisen concerning how to interpret data and where to go next.

It is always of interest to ask why one is doing a certain type of study. Probably the first reason for anyone in scientific work is just plain curiosity. Second, the quantification of a system may lead to the understanding of the mechanisms that control it and, through studying the influence of perturbation as has been done so well with erythropoiesis, one learns much about the regulation of the system. Third, understanding the normal way may give better insight into the abnormal, in this case, the leukemic process. Finally, and probably of most importance, is that understanding the general field may lead to an entirely fresh outlook on cell proliferation and its aberrations. I think the time is right to ask whether chemotherapy is in a rut because of poor concepts and whether the quest for the virus is really as futile as searching for the Holy Grail in the Middle Ages. I say none of these things in any sense of criticism, but I do think that, with the state of knowledge today, one must reappraise the motivations behind the various studies.

My specific comments will be on the autoradiographic procedure following the administration of ^{3}H-TDR to human beings and, to a limited extent, on the enumeration of mitosis in various compartments within the bone marrow. This work has been done jointly with Drs. Fliedner, Bond, Killmann, Rubini, Robertson, and Stryckmans since 1957 (*7–9*, *23–33*).

Text-figure 1 presents a schema of granulocytopoiesis. The general concept states that a stem cell pool exists, the cytologic identity of which is still obscure. In some manner inductors act on this pool and induce either the common or specific stem cells down various differentiated pathways, such as granulopoiesis, erythropoiesis, or megakaryocytopoiesis. Once committed to the differentiated proliferating pool, the cells continue to differentiate and go through successive divisions. Later there is an important transition from the differentiated proliferating pool to a nonproliferating pool in which maturation continues. Still later the cells attain a degree of maturity in which they are able to leave the bone marrow and enter the blood as a functional pool of granulocytes, and then ultimately a series of death processes takes place. The three important transitions are: 1) the stem cell transition, 2) the transition from the dividing to the nondividing pool, and 3) the transition from the nonproliferating pool into the functional pool of cells. The latter two are easily discernible. The first is unknown, since the identity of the stem cell has not yet been made, so one cannot determine the flux from this pool into the granulocytopoietic, erythropoietic, or megakaryocytopoietic pools. This may only be inferred.

Text-figure 2 shows the cellular generative cycle consisting of a rest period after mitosis (G_1), a period of replication of the chromosomes in which precursors of DNA will be incorporated into the chromosomes (S), another rest period (G), and then mitosis. Actually, in most hematopoietic cells G_1 is *very* short or almost absent. After labeling, this block of labeled



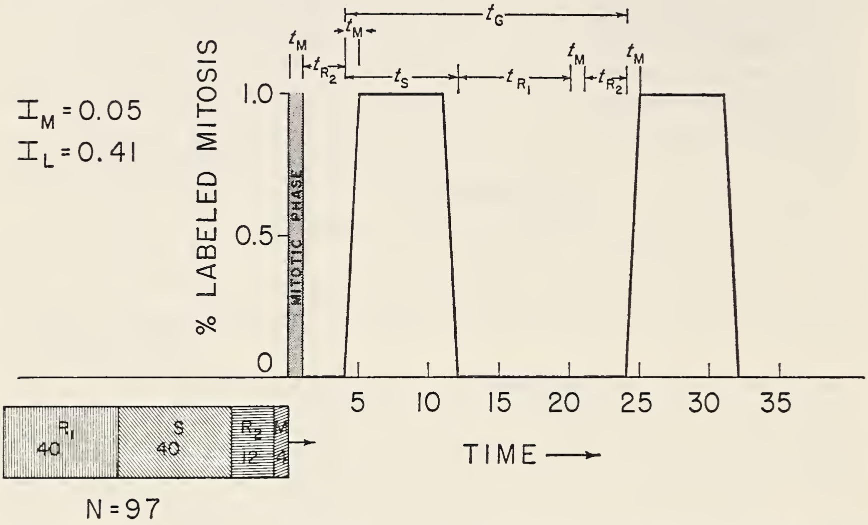
TEXT-FIGURE 1.—The flow of cells through successive cytologic compartments from the stem cell. (Brookhaven National Laboratory #11-292-68.)

cells moves through G_2 and through mitosis. Ony the cohort of labeled mitoses moves through mitosis, and one can deduce much about the duration of the various segments of the generative cycles (text-fig. 2).

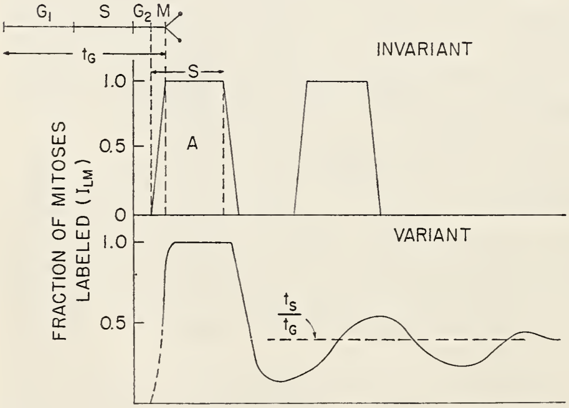
Text-figure 3 shows that, if the rate of proliferation is constant, the labeled cells will appear in mitosis after a period equal to mitosis and G_2, go up to 100%, remain at 100% until the end of the DNA synthesis, drop again, and keep repeating this cycle if there were no variance in the system. If the speed of proliferation varies, as in living systems, one will see the first wave coming through, and depending on the degree of variance, one may see a second wave or it may become damped out very quickly to the ratio of the synthesis time to the total generation time.

Text-figure 4 gives examples of labeled mitosis curves that were obtained in humans both for erythropoiesis and granulocytopoiesis. In our hands, these curves usually attain 100% labeling. There is always a drop, but it has never returned to zero labeling between first and second wave. Sometimes we see a second wave; sometimes it becomes damped out immediately, or perhaps we do not have sufficiently frequent samples to pick up a definite second peak.

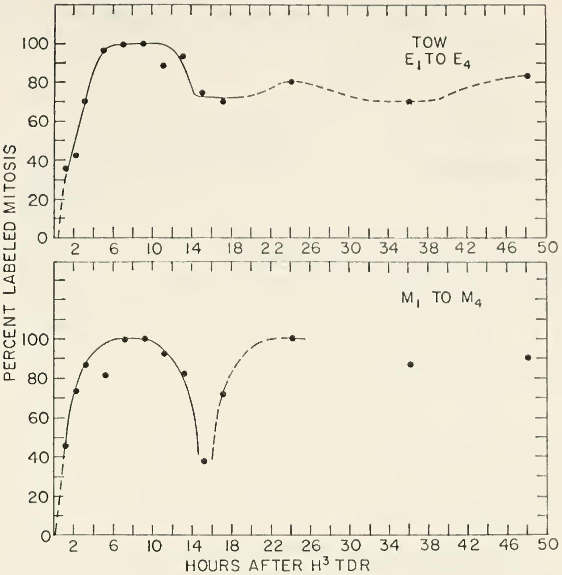
Text-figure 5 represents another study of both granulocytopoiesis and erythropoiesis. At the beginning and end of DNA synthesis, the cell have less opportunity to incorporate ^{3}H-TDR because they are in synthesis for only a short period while ^{3}H-TDR is available. One should see then, as time passes, lightly labeled cells coming into mitosis, then more heavily labeled cells, followed by more lightly labeled cells. The time between the first labeled and the last lightly labeled cells should approximate the maximum synthesis time for the individual studied.



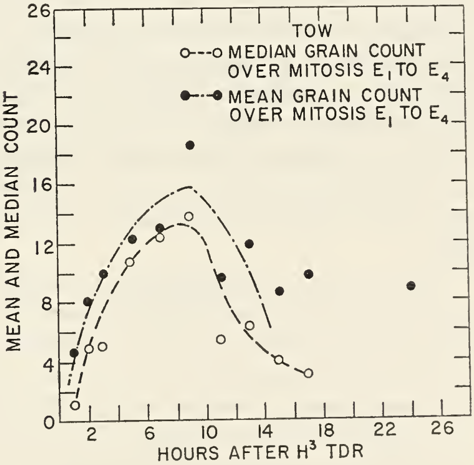
TEXT-FIGURE 2.—Cells labeled during DNA synthesis moving through the first and successive mitoses and showing changes in the fraction of mitotic figures labeled. In a system in which there is no variance of the generative cycle or its subdivisions, there is no curvature. (Brookhaven National Laboratory #4-343-61.)



TEXT-FIGURE 3.—The introduction of biologic variance of the generative cycle and its subdivisions rounds out the labeled mitosis curve. (Brookhaven National Laboratory #8-515-64.)



TEXT-FIGURE 4.—An example of labeled mitosis curve in bone marrow from a human given ^{3}H-TDR. E: erythropoietic mitosis. M: granulopoietic mitoses. [Stryckmans *et al.* (*33*).] (Brookhaven National Laboratory #11-91-64.)



TEXT-FIGURE 5.—Time trend in the mean and median grain counts overlying erythropoietic and granulopoietic mitoses. [Stryckmans *et al.* (*33*).] (Brookhaven National Laboratory #11-88-64.)

Table 1 tabulates DNA synthesis times obtained to date from study of human bone marrow. An estimate for the minimum DNA synthesis time for erythropoiesis is 11 hours and for myelopoiesis 13 hours. The average time is estimated in the column, with a somewhat shorter minimum and average time for DNA synthesis for erythropoiesis as compared to granulocytopoiesis. This may be critical, as will be discussed later in making computations.

TABLE 1.—Tabulation of estimates for DNA synthesis time*

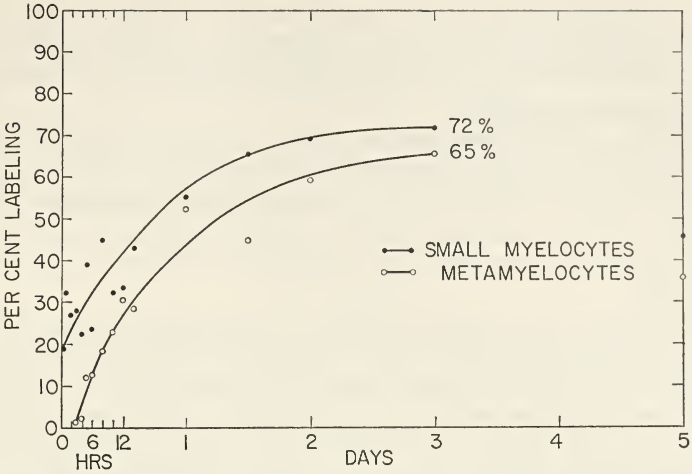
Patient	Cells	DNA synthesis time	
		Minimum	Average
Normal blood 1	E_4	11	>12
	M_1–M_3	13	>13
	M_3	13	>13
Normal blood 2	E_1–E_4	11	13
Packed cell volume	E_1–E_4	11	13
	M_1–M_4	11	13
Myeloid metaplasia	E_3	12	14
	E_4	~16	?
Myeloid metaplasia	E_3, E_4	13	?

*Stryckmans *et al.* (*33*).

One relatively easy study in our initial work was to observe the intensity of the label over various cytologic classes in the bone marrow at different intervals after the administration of ^{3}H-TDR. At the present time the use of grain counts for estimating generation time is highly unreliable, or at least uncertain, because it will only represent the generation time if each cell that comes into the given cytologic class divides in the class. If there are no reutilization of the labeling materials and no influx of heavily labeled cells from an earlier compartment, all the preceding three factors would decrease the rate of diminution in the intensity of the label. It is not feasible to answer these questions quantitatively now. The half-time for grain count diminution can only give a crude estimate of the upper limits for generation times and probably will always overestimate the generation time.

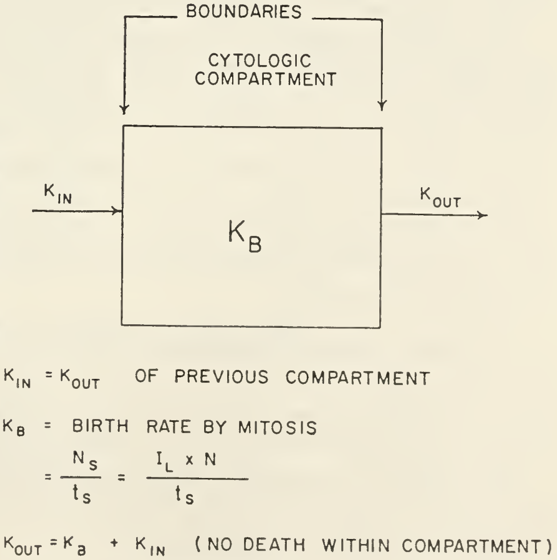
Next there is a morphological problem. The smaller and larger myelocytes have a different kinetic pattern. The "flash" labeling of the smaller class is much lower, around 10–20% (*29*), whereas the labeling index in the larger class is much higher, around 70%. With time, the percent labeling in the smaller cells increases to that of the larger myelocytes by the 3d day. This indicates that the growth fraction of the myelocyte is about 30–40%. Label appears first about 3 hours after injection, setting a minimum for the transit from the myelocyte to the metamyelocyte, and then increases (text-fig. 6).

The rate of transition from the proliferating compartment to the nonproliferating compartment furnishes an estimate of the turnover of the first part of the nonproliferating compartment. In the metamyelocyte in this patient, it is about 3.3% per hour on the steep slope of the curve. There has been some variability from patient to patient. The range so far has been about 3–5% but we have had inadequate samples for a good estimate of mean and variance.



Text-figure 6.—Comparison of changes in the labeling index between the small Myelocytes and the metamyelocytes. (Brookhaven National Laboratory #11-207-59.)

Within a given cytologic compartment one can measure the birthrate by mitosis from the simple relationship of the relative number of cells found in synthesis in that compartment divided by the time for synthesis of that specific cell type. From the ordinary marrow differentials and the fraction of every cell type that is labeled, one calculates the number in synthesis (N_s) and this divided by the synthesis time gives the birthrate by mitosis. The cells going out are equal to the cells coming in, plus the cells born within the compartment, making the assumption that there is no death within this compartment. This is illustrated in text-figure 7.


BOUNDARIES
CYTOLOGIC COMPARTMENT
K_{IN}
K_B
K_{OUT}
$K_{IN} = K_{OUT}$ OF PREVIOUS COMPARTMENT
K_B = BIRTH RATE BY MITOSIS
$= \frac{N_S}{t_S} = \frac{I_L \times N}{t_S}$
$K_{OUT} = K_B + K_{IN}$ (NO DEATH WITHIN COMPARTMENT)

Text-figure 7.—Estimation of the flux of cells into the birthrate in, and the flux out of, a cytologic compartment. N_s: number of cells in DNA synthesis. t_s: DNA synthesis time. (Brookhaven National Laboratory #6-481-64.)

Patt and associates (*34–37*) have described ineffective granulopoiesis with substantial cell death at the myelocyte level in the dog. For reasons that are unclear to me, we have not observed this in the few patients with normal hematopoiesis (*27*).

However, in pernicious anemia there is marked ineffective granulopoiesis (*27*). Transit through the nondividing compartment is reasonably well established. It takes a minimum of 3 hours for the first metamyelocytes to become labeled, 12 hours for the label to appear in the juveniles, roughly 36 hours in the band cells, and about 48 hours in the segmented neutrophils.

The emergence time of granulocytes into the peripheral blood is defined as the time from injection of ^{3}H-TDR until the first labeled granulocytes are observed in the peripheral blood. This has been discussed in detail by Fliedner *et al.* (*31*, *32*). The best approximation of the emergence time in the normal steady state is somewhere between 96 and 144 hours. In an individual[2] studied 3 successive times—once in a normal steady state, once with a mild urinary-tract infection, and later with an acute lobar pneumonia—the emergence time progressively decreased to 72 hours with the mild infection and 48 hours during an acute lobar pneumonia. These scattered observations suggest the emergence time is significantly reduced by the presence of acute bacterial infection.

An old observation in hematology is a small number of pyknotic granulocytes in the peripheral blood under normal circumstances. The ratio of pyknotic to nonpyknotic cells varies from 1 in 500 to about 1 in 2,000 cells. If this represents a senescent process in granulocytes, one should be able to measure the time for this process by the appearance of label in the segmented pyknotic cells. This has been observed in several patients by Fliedner *et al.* (*31*). There is a uniformly constant 24–30 hours before label begins to appear in the pyknotic segmented neutrophils.

The excellent studies of the Salt Lake City group (*19–22*) have thoroughly established that the loss of granulocytes from the peripheral blood is random with a half-time of about 6.6 hours. To confirm random loss directly, it appeared desirable to obtain direct evidence of the loss of labeled cells from the bloodstream simultaneously with their appearance in the bloodstream. This was done by making oral lavages and autoradiographs of the cells sedimented from the washings. Almost simultaneously with the appearance of labeled cells in the blood, labeled cells were found in the oral washes (*32*).

We believe that the granulocytes are lost from the peripheral blood by two processes. The first process is a random loss with a half-time of about 6.6 hours. This random loss is truncated by a senescent process at about 30 hours, resulting in the formation of pyknotic cells that are lost from the blood with an estimated half-time of about 15 minutes.

The notion of a senescent process truncating the random loss has been questioned in principle. To get direct evidence for such a loss, we utilized identical twin calves. One twin was labeled with ^{3}H-TDR and, at the time of maximum labeling of granulocytes, cross-circulated with its twin. On completion of cross-circulation, labeled cells in the unlabeled twin disappeared. By 28 hours after cross-circulation, all granulocytes had disappeared from the peripheral blood.[3] These studies are continuing; however, this single observation appears to be a direct confirmation of a maximum lifespan for the labeled granulocytes.

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[2] Comatose from glioblastoma multiforme.

DISCUSSION

The rate of flow of cells through the proliferating granulopoietic compartments is reasonably well established. The minimum times for transit through the nondividing compartments are quite well determined. The time for emergence of granulocytes from the stage of labeling in the marrow to their appearance in the peripheral blood is well established. The question of DNA synthesis time is vital and the calculation of relative production depends on this parameter. The failure to confirm in man an ineffective granulocytopoiesis, as found by Patt and associates in the dog, may be due to the use of an unduly long DNA synthesis time. In addition, one should be able to estimate birthrates from mitosis and from synthesis time and, clearly, these should be equivalent. Unfortunately, either because of large inherent error in estimates or technical reasons not clear now, this has been impossible in our studies on man. The problem is not trivial, since a concept on a partial regulation of the system has been proposed by Patt *et al.* in which it is believed that death at the myelocyte level is part of the regulatory mechanism.

Conceivably, the methods by which the mitotic time and the DNA synthesis time are calculated are too insensitive to determine the time with sufficient precision to detect a 10–20% ineffective granulopoiesis.

In the steady state, the birthrate equals the death rate of cells. One can easily calculate the death rates from total number of cells in the peripheral blood and the known lifespans. Birthrates can be calculated as follows:

$$K_{BE} = \frac{TMC \times f_E \times f_{SE}}{t_{SE}} = \text{birthrate red cells.} \qquad [1]$$

$$K_{BG} = \frac{TMC \times f_G \times f_{SG}}{t_{SG}} = \text{birthrate granulocytes.} \qquad [2]$$

$$[1] \div [2] = \frac{K_{BE}}{K_{BG}} = \frac{f_E \times f_S}{f_G \times f_S} = \frac{N_{SE}}{N_{SG}}$$

providing that $t_{SE} = t_{SG}$.

TMC = total marrow cellularity
f_E = fraction in erythropoiesis
f_{SE} = fraction labeled in erythropoiesis
f_G and f_{SG} the same for granulopoiesis
N_{SE} = number erythroid cells in DNA synthesis
N_{GS} = number granulocytic precursors in DNA synthesis

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[3] Unpublished observations by A. D. Chanana and E. P. Cronkite.

The preceding indicates that the ratio of the birthrates of erythropoietic cells to granulopoietic cells equals simply the ratio of the number of erythropoietic cells in synthesis to the number of granulopoietic cells in DNA synthesis, since total marrow cellularity time and DNA synthesis time cancel out. However, the synthesis times for erythropoiesis and granulocytopoiesis must be identical.

Since one only has to do careful bone marrow differentials and flash labeling of the bone marrow to determine the number of erythropoietic cells in synthesis and the number of granulopoietic cells in synthesis, in principle it should be easy to determine if the birth and death rate ratios are equal.[4] If they are equal or not significantly different from each other, the idea of ineffective granulopoiesis or erythropoiesis can be eliminated. If they are significantly different, one must then conclusively demonstrate that granulopoietic and erythropoietic DNA synthesis times are equal; if the latter is not established, the question of ineffective granulopoiesis and erythropoiesis must be held in abeyance.

Another factor that must be considered is the question of a neutrophil cycle in normal individuals. Morley *et al.* (*38*) have published data suggesting normal neutrophil cycles of 14–23 days with oscillations between 2000 and 4000 per mm^3. He interprets these observations as implying the existence of a negative feedback circuit acting on the marrow at the level of the proliferative pool. Whether the probable cycling is due to changing generative cycle times or to cyclic inputs of stem cells is not established. In any case, it appears risky to base birthrates of neutrophils on single marrow observations. To determine marrow production daily throughout a whole cycle in man is impractical. At this stage one must be hesitant to interpret equality in birth and death ratios of erythropoietic and granulopoietic cells as signifying absence of ineffective hematopoiesis in one cell line or the other, or that inequality proves presence of ineffective hematopoiesis.

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Possible Significance of Nonproliferating Leukemic Cells[1]

Alvin M. Mauer,[2] E. F. Saunders,[3] *and* Beatrice C. Lampkin,[4] *Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati, Cincinnati, Ohio 45219*

At a recent meeting of the Leukemia Society, Dr. Cronkite said he once thought the study of cell kinetics in relationship to acute leukemia had much worth in terms of therapy, but since then he has become very discouraged and feels that actually cell kinetics have not offered much of anything. I agree with his opinion on the application of cell kinetics to conventional therapy, but I do think that studies of cell kinetics in acute leukemia have at least given us some potentially important insights about leukemia. I would like to develop this theme.

One ought to start off by saying what one would anticipate from the study of cell kinetics in acute leukemia. Of early interest was an attempt to explain the growth differential between leukemic cells and normal cells. After all, a situation exists in which a normal marrow population, which is itself dividing, is replaced by another dividing cell population. The first concepts were that the abnormal cells were dividing more rapidly. These cells were described in such terms as "wildly proliferating." If we had paid attention to the studies by Astaldi and Mauri (*1*), we would have abandoned this concept a long time ago. By looking at the mitotic index of the leukemic cell population, these investigators were first to point out that in fact the leukemic cell population was not a wildly proliferating population. With time, our concepts have grown. We now have some answers as to whether the accumulation of cells is just that, an accumulation, or whether it is a more rapidly proliferating population.

A second problem I think we can tackle is to develop some concept as to the pathogenesis of the leukemic cell process: In particular, we can attempt to ascertain what the source of the leukemic cell is, with reference to whether there is a continuing input of transformed normal cells during the leukemic process or whether this is a self-contained, self-replicating population of cells.

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[2] A recipient of a research career development award from the National Institutes of Health.

[3] Supported by the Leukemia Society, Inc.

[4] Advanced Clinical Fellow of the American Cancer Society, Inc.

Finally, despite Dr. Cronkite's reservations, we have learned some things that give us at least guidelines to future considerations for therapy. These are the three aspects to be considered as far as the contribution of kinetics is concerned. I think there have been contributions in all three areas.

A brief review of the tools that we have used to study cell kinetics in acute leukemia follows. We have studied only children with acute leukemia. We have no data on chronic leukemia or on acute leukemia in adults.

The leukemic population, since it is a dividing population, gives us two opportunities to measure proliferative activity. After mitosis, the cell enters an interphase during which time little activity referable to cell division takes place. Then there is a time of DNA duplication when the proliferating cell can be identified by tritiated thymidine (^{3}H-TDR) incorporation. Then there is a postsynthesis rest phase followed by mitosis. During mitosis, since this is now a structurally identifiable cell, we have one other marker as an index of proliferative activity.

The incorporation of ^{3}H-TDR does something other than merely identify a section of the proliferative phase. It also allows us to tag a cohort of cells which will subsequently go through mitosis, dividing the label equally between daughter cells. We can then follow this population of cells, either through another cell division or from one compartment, such as bone marrow, into another, such as blood. So the ^{3}H-TDR label has been particularly important. With autoradiography, we can identify morphologically the labeled cell. We can also get some idea as to the quantity of label within the cell by counting the number of overlying silver grains.

The percentage of mitotic figures can be determined by a modification of the method of Japa (*2*). Marrow particles are put in acetocarmine solution and squashed between coverslips and slides. In this preparation, one can count the number of nucleated cells, which then have been cleared of red cells, and determine the number of mitotic figures. One thousand nucleated cells are counted from each of 10 coverslips. The results are expressed as the number of mitotic figures per 1,000 nucleated cells.

Before we consider applying these methods to the study of acute leukemia, we must raise some important questions. If we want to do serial determinations of marrow proliferative activity or if we want to be sure that a single sample of marrow is representative of the marrow activity in that patient, we must determine whether proliferative activity is uniform throughout the marrow spaces. To study this question we injected ^{3}H-TDR intravenously. One hour later we took 6 marrow samples over a 15-minute period and determined the labeling index with ^{3}H-TDR (the percentage of cells incorporating ^{3}H-TDR) and the amount of label per cell (*3*).

The results of this study are shown in table 1. The 6 sites of marrow samples did not differ significantly. Therefore, a marrow sample does represent the general marrow proliferative activity. Another problem we have is that, although some studies, such as this one, are done *in vivo*, for convenience other studies are done *in vitro* so that a marrow sample can be obtained and incubated with ^{3}H-TDR in order to acquire a labeling index as a measure of proliferative activity. Then, does an *in vitro* labeling index give us the same information as an *in vivo* study?

TABLE 1.—Distribution of label in the bone marrow

Minutes after injection	Site of aspiration	Labeled blasts (%)	Mean grain count
60	Vertebral spine	15. 6	13
61	Vertebral spine	12. 4	11
62	Vertebral spine	15. 7	12
63	Vertebral spine	15. 9	14
65	Left anterior iliac crest	15. 0	15
75	Right anterior iliac crest	15. 5	14

The results of three such studies are presented in table 2 (*4*). For these studies, marrow samples were obtained from 3 patients and incubated for 1 hour with ^{3}H-TDR. As soon as these samples were obtained, ^{3}H-TDR was injected intravenously. One hour thereafter a second marrow sample was obtained to determine the *in vivo* labeling index. In these 3 patients equivalent results were obtained. Therefore, results obtained from such an *in vitro* technique reflected the *in vivo* situation.

TABLE 2.—Correlation of 1-hour *in vivo* and *in vitro* labeling of leukemic cells

Patient	*In vivo*	*In vitro*
1	11. 8	12. 6
2	4. 4	5. 5
3	5. 0	4. 1

Of immediate concern in the kinetics of acute leukemia was how the dividing leukemic cell population overgrows normal cells—again returning to the concept of a wildly proliferating population of cells. One of the foci of interest was a study of generation time, if one anticipates that the generation time would be very much shorter. Generation time has to be considered apart from doubling time, since it is equal to doubling time only if the entire cell population is dividing. If a fraction of cells is nondividing, then generation time will be shorter than the doubling time.

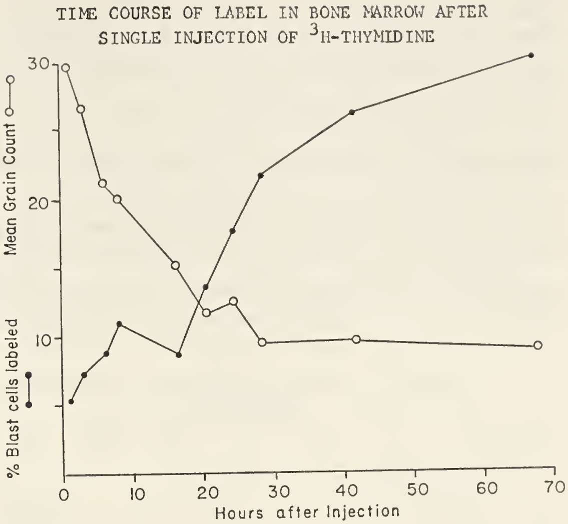
The first attempt was simply to obtain the cells, incubate them with ^{3}H-TDR, and determine a labeling index (*5*). It was assumed that all cells were dividing and were randomly distributed in the mitotic cycle. Therefore, a measure of the cells in DNA synthesis, assuming a known DNA synthesis time, would give the generation time for the population. These studies gave an extraordinarily long generation time because of the low-labeling index. Since that time, it has become clear that the leukemic cell population is in fact not uniform as to proliferative activity, and that this method is therefore not valid.

The next approach was to measure the rate of decreasing grain count. Since each time the cell divides it halves its grain count, one could then use a grain-count-halving time to indicate generation time (*6*). This raises technical problems. One problem is that some cells which have few grains will divide one time and have so few grains left over for each daughter cell that they cannot be differentiated from background. These cells are then lost. A second problem is that label reutilization artificially increases the mean grain count. A third problem is that, if some cells divide but then stop dividing, their grain count will not change. Therefore, the accumulation of nondividing cells will again artificially hold the mean grain count up. So, for these three reasons, the rate of decrease in mean grain count has not proved to be a valid assessment of generation time.

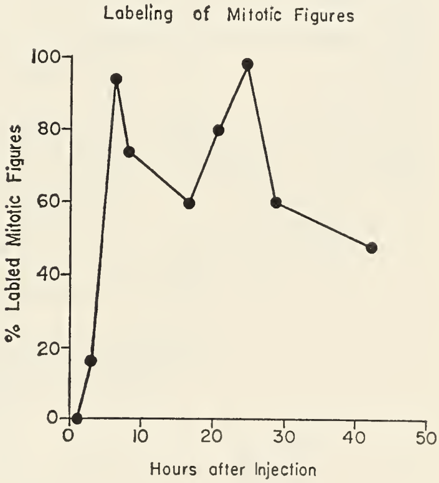
Another approach was to look at the time course for percentage of labeled cells after an injection of ^{3}H-TDR. Text-figure 1 gives the results from a study of a patient with acute lymphoblastic leukemia. After the injection, about 6% of the cells were labeled. Subsequently the percentage of labeled cells increased until about 10 or 12 hours. Then about twice the number of cells were labeled, as apparently this cohort of cells had gone through division, producing twice the number of daughter cells. Beginning again at 20 hours, the percentage of labeled cells again increased, redoubling the number of cells seen as a result of this second division. During both increases in labeled cells the mean grain count decreased. We concluded that, in this particular patient, the generation time from division to division was about 20 hours. Interestingly, this study demonstrated that with each division the percentage of labeled cells increased as if these labeled cells were increasing in a population of nondividing cells.

Text-figure 2 shows the results of the labeling of mitotic figures from this study. The percentage of labeled mitotic figures increased by 6–10 hours. Then it decreased, as apparently unlabeled cells completed DNA synthesis and went through mitosis. Subsequently, at 20 hours, it again increased, indicative of a generation time of about 20 hours in this patient.

Another approach we took is shown in text-figure 3. It involves 3 serial injections of ^{3}H-TDR at 10-hour intervals. After the first injection, we found about 15% of the cells in the bone marrow were labeled. After the second injection, we found another increase. At 20 hours, we injected the label, but the percentage of cells labeled did not change, although the mean grain count increased markedly, indicating that at this time the cells that took up the newly supplied label were already labeled. In this patient likewise we found a generation time for the dividing cells of about 20 hours.



Text-figure 1.—Labeled blast cells after *in vivo* labeling with ³H-TDR.

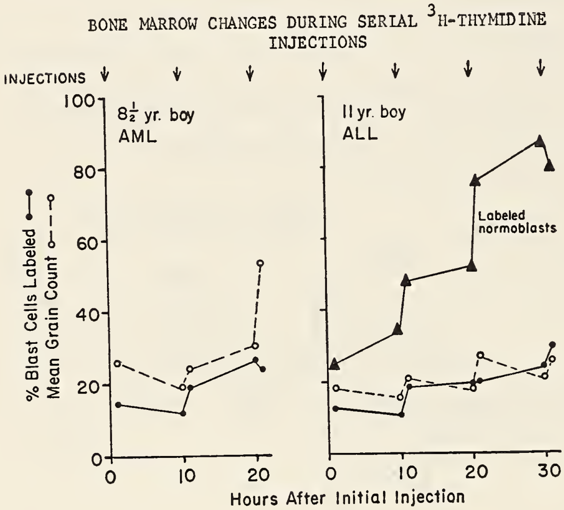


Text-figure 2.—Time course for labeled mitotic figures after *in vivo* labeling with tritiated thymidine.

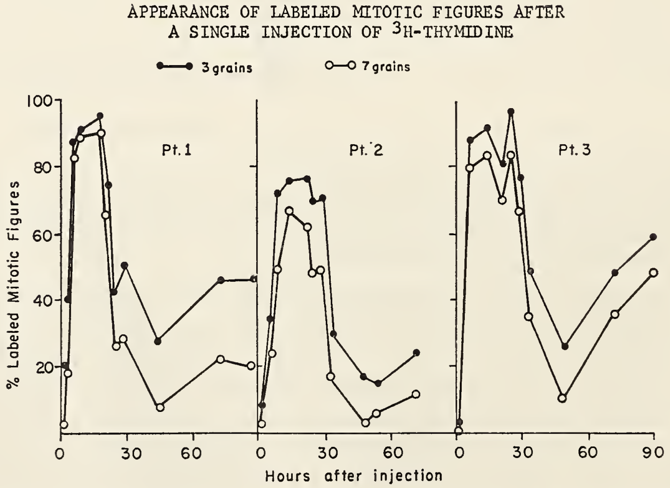
Once again, although we seemed to have saturated the dividing cell population fairly well, still less than 30% of the cells were labeled, indicating that many of these cells were nondividing.

A study, using what Dr. Cronkite has called the "mitotic window," has already been shown. The results in 3 further patients are shown in text-figure 4. This technique involves the injection of the label and subsequent determination of the percentage of labeled mitotic figures. In these 3 patients, initially there were no labeled mitotic figures at 1 hour. Subsequently, the percentage of labeled mitotic figures rapidly increased and then rapidly decreased as those cells in DNA synthesis that were labeled went through mitosis and appeared as labeled mitotic figures. These cells finished mitosis and were replaced by the cells entering the DNA synthesis phase after the label had been made available in the flash-labeling technique. In these 3 patients, the curves were similar. Labeled mitotic figures once again appeared at about 60 hours, indicating that these cells, having gone through mitosis, were again appearing in mitosis within 60 hours of the injection. Therefore, in these 3 patients the generation time was approximately 60 hours for dividing cells.

The results of these studies have been essentially the same as those found in animal tumors (*7, 8*) and in human tumors (*9*) ; *i.e.*, the time required for the various phases of the generation cycle by the malignant cells has been the same as or longer than that of the normal tissue. In our



TEXT-FIGURE 3.—The study referred to in the text is presented in *left panel.* The study in *right panel* is similar.

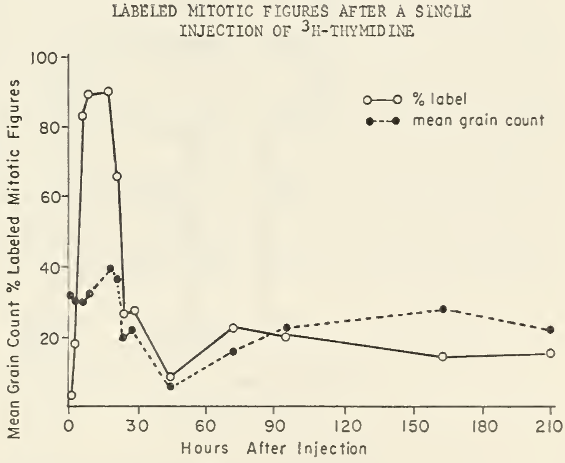


TEXT-FIGURE 4.—Time course for labeled mitotic figures in 3 patients.

results so far the times range from 20–60 hours for the dividing leukemic cell. This observation certainly cannot explain the leukemic cell growth differential because the cells are not dividing more rapidly. They are either dividing as rapidly as or even less rapidly than the normal cell population.

One other important point is that apparently generation time is not the same for all cells within the population.

Shown in text-figure 5 are the results of a study on a patient from whom we obtained marrow samples for 210 hours after the injection of the label. Cells have 7 grains or more, to reduce the factor of label reutilization. Initially, as we have seen before, there was a very well-defined wave of labeled mitotic figures. A second wave of labeled mitotic figures began at about 60 hours. At that time the mean grain count of the labeled mitotic figures was about half of the initial mean grain count, indicating that these mitoses were indeed second generation. However, even as late as 210 hours, labeled mitotic figures were found having a mean grain count about the same as that seen with the second generation. In this patient, some cells were redividing with a 60-hour generation time, whereas other cells, which apparently had a long interphase, did not divide until 210 hours after the labeling period. Therefore, within the same population there may very well be considerable variation of generation time.

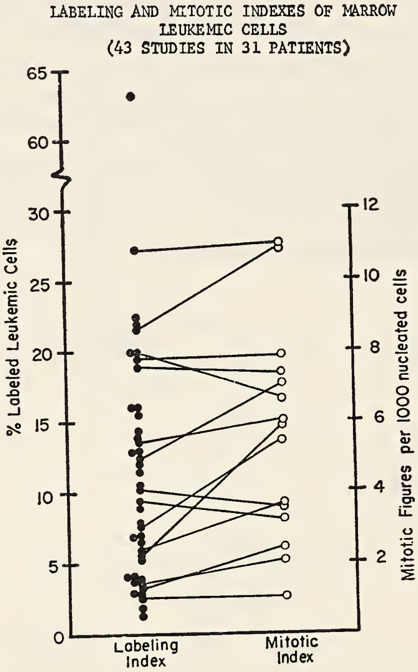


Text-figure 5.—Time course for labeled mitotic figures followed for 210 hours.

Another striking feature of studies in acute leukemia has been the variability of proliferative activity that we have seen in various patients. Text-figure 6 shows the results of studies of labeling and mitotic indexes in 31 patients in whom 43 studies were available. The agreement between the labeling index and the mitotic index as an indication of proliferative activity was good. The labeling and mitotic indexes that were obtained in the same patient are connected by lines in the text-figure. The labeling index ranged from 1–63%, the mitotic indexes from 1/1,000 to 11/1,000.

There was considerable variation from patient to patient and in the same patient during different stages of the disease.

To what was this variation of proliferative activity related? A clue came from early observation of the labeling of bone marrow and blood leukemic cells (table 3). The labeling index of bone marrow, compared to that of blood, is found in every case to be greater (*10*). The bone marrow therefore appears to be the primary proliferative compartment, the blood containing primarily nonproliferative cells. With the indication that there might be a nonproliferating blood compartment, we can look back at the bone marrow and see whether we can differentiate proliferative and nonproliferative compartments there also.



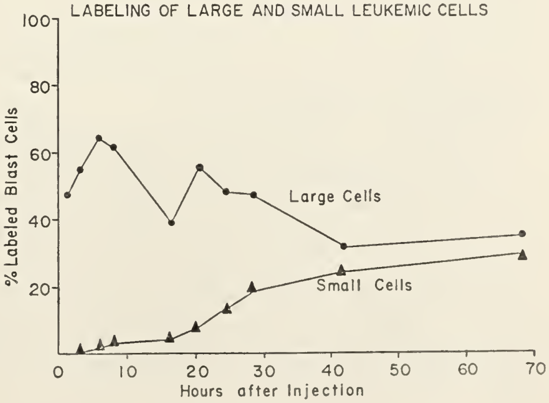
Text-figure 6.—Proliferative activity measured in 31 patients.

Table 3.—*In vivo* labeling of leukemic blast cells

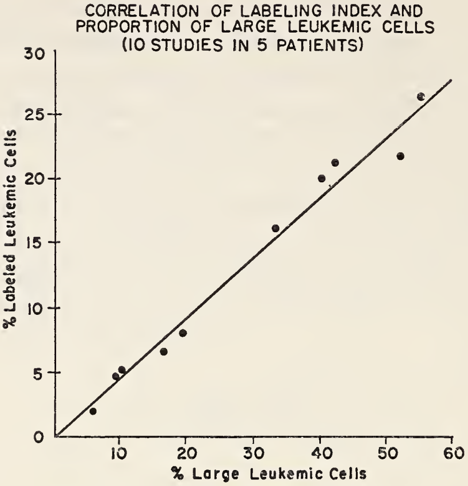
Type of leukemia	Percentage of labeled cells	
	Bone marrow	Blood
1. Acute lymphoblastic	2. 6	0. 3
2. Acute lymphoblastic	3. 0	0. 5
3. Acute lymphoblastic	7. 5	1. 0
4. Acute myeloblastic	26. 4	4. 9
5. Chronic myelocytic	47. 5	5. 5

Figures 1 and 2 and figures 3 and 4 are autoradiographs obtained 1 hour and 24 hours, respectively, after the injection of ^{3}H-TDR. Gavosto *et al.* (*11*) and Killman (*12*) have also reported that, 1 hour after injection, the only cells labeled were the large leukemic cells that had rather fine nuclear chromatin patterns. The small leukemic cells with a denser chromatin pattern were not labeled. However, in the marrow sample seen 24 hours later (figs. 3 and 4), label then appeared in the small cells.

In text-figure 7 are shown the results in one of our patients demonstrating the change in percentage of labeled cells in the large cell compartment and in the small cell compartment. Indicated is the percentage of labeled blast cells. In this case, the large cells represented about 12% of the leukemic cell population of the marrow, and this remained the same throughout the study. About 50% of these cells were initially labeled, but this percentage decreased in the next 24–48 hours. As it decreased, the percentage of labeled small cells increased as some labeled large cells divided to become small leukemic cells. Thus the large cell compartment is related to the small cell compartment in that the former is the proliferative compartment which feeds into the small cell compartment—very much like a pronormoblast feeds into the erythrocyte and the myelocyte feeds into the metamyelocyte and polymorphonuclear leukocyte. In fact, if one takes patients who have varying labeling indexes and compares their indexes to the proportion of the large cells as shown in text-figure 8, the relationship between the percentage of labeled leukemic cells and the percentage of large leukemic cells is fairly close. Therefore, the changes in labeling index are related primarily to changes in the proportion of large dividing cells. Actually, the labeling indexes of the large cells have been rather constantly about 50%, which is not far removed from the labeling indexes of normal marrow cells obtained by Cronkite and Fliedner (*13*). The large leukemic cell seems to proliferate in a relatively normal fashion, but it feeds into a not really functionally maturing small cell leukemic fraction.



TEXT-FIGURE 7.—Time course for labeled large and small cells.

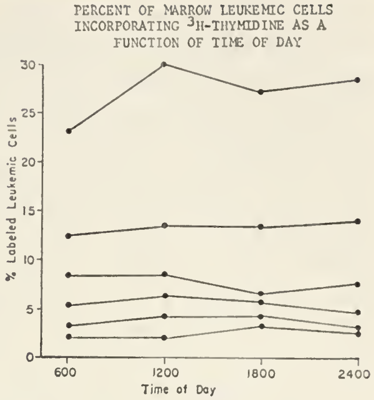


TEXT-FIGURE 8.—Labeling indexes and proportion of large blast cells.

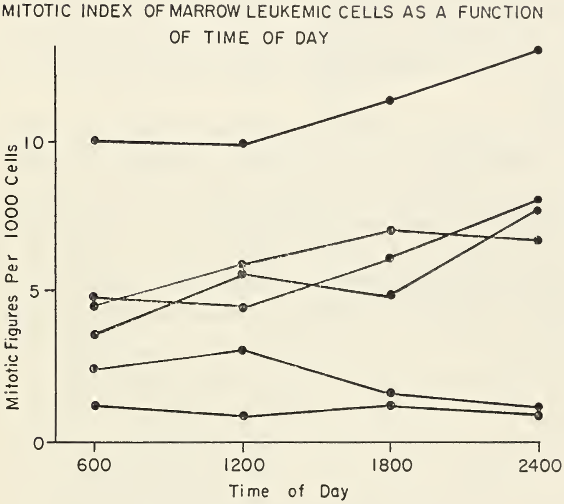
These studies have another important aspect. Variation, of course, of the proliferative activity has to concern itself also with possible circadian effects on cell proliferation. Text-figure 9 shows the results of studies done on 6 patients from whom marrow samples were obtained at 6:00 AM, 12:00 AM, 6:00 PM, and 12:00 PM. Labeling indexes were also obtained at these times, and in these patients labeling indexes did not vary significantly throughout the day. This has some relevance in terms of therapy. Therapy affecting DNA synthesis primarily would optimally be given at the time of maximal DNA synthesis. In the leukemic patients we have studied, there would seem to be no optimal time of day for this type of therapeutic agent.

The mitotic indexes obtained at the same time in these patients are shown in text-figure 10. Interestingly, in 2 of these patients with very low mitotic indexes, the indexes did not vary significantly. In the other 4, however, at 12:00 PM or 6:00 PM, the mitotic indexes were significantly higher than those obtained at 6:00 AM. This is also found in normal marrow mitotic indexes (*14*), which indicates that these abnormal cells respond, at least partially, to some of the normal control mechanisms involved in cell proliferation within the marrow.

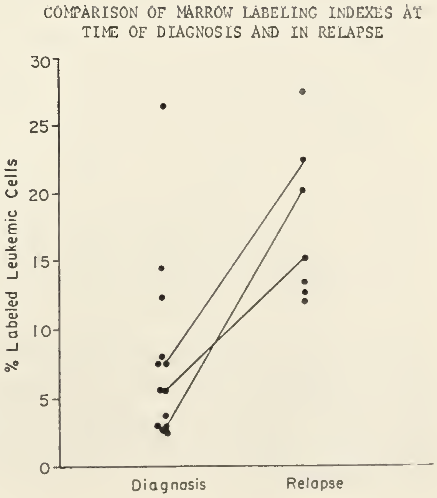
We found one interesting correlation of proliferation with disease. But we could not correlate the following features with proliferative activity: age of the patient, sex, type of leukemia, degree of marrow replacement, the blood leukemic cell count, or the duration of survival after the marrow sample was obtained. However, the labeling indexes obtained at diagnosis and at relapse were different (text-fig. 11). Those obtained at diagnosis were significantly lower than those obtained at relapse. Most patients at the time of diagnosis have had symptoms for some time, and most patients have had growth of their leukemic cell population for some time



Text-figure 9.—Labeling indexes obtained in 6 patients at 6-hour intervals.



Text-figure 10.—Mitotic indexes obtained in 6 patients at 6-hour intervals.



Text-figure 11.—Labeling indexes at diagnosis and in relapse. Studies in the same patient are connected by *lines.*

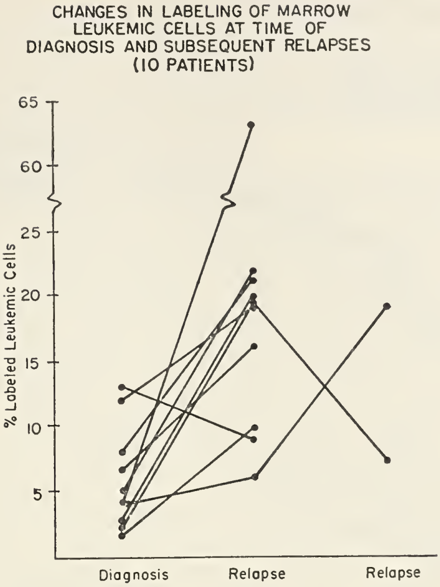
before admission to the hospital and study. On the other hand, patients who were studied in relapse were followed rather closely. As soon as relapse was noted, either symptomatically or by changes in the blood count, a marrow sample was obtained. One would presume that these cells had been growing for a shorter time than the former cell population. Furthermore, in all those patients in whom the labeling indexes were obtained at diagnosis, all those who had labeling indexes greater than 6% had symptoms less than 2 weeks. This is interesting because there are now several animal tumor models in which a progressive change in the fraction of cells participating in cell proliferation occurs with growth of the tumor (*15–18*). As the tumor grows, nonproliferative cells accumulate progressively. From these data in leukemic patients, the same process might occur; *i.e.*, with a longer time of population growth, nonproliferative cells accumulate, which accounts for the low labeling indexes. We have recently tested this directly in one patient from whom we were able to obtain 3 marrow samples within 2 weeks. His labeling index decreased progressively during this period of study.

Text-figure 12 shows the changes in labeling index within the same patient at diagnosis and subsequent relapses. Again we have the same phenomena. In 8 of 10 of these studies, the fraction of dividing cells increased, either as indicated by the portion of large cells or the labeling index from diagnosis to the subsequent relapse. Text-figure 13 shows a comparison of labeling index and proportion of large blasts from 4 patients. As the labeling index increased or subsequently decreased, the proportion of large blasts changed. In these patients, this variation was related to changes in the proportion of the dividing cell population.

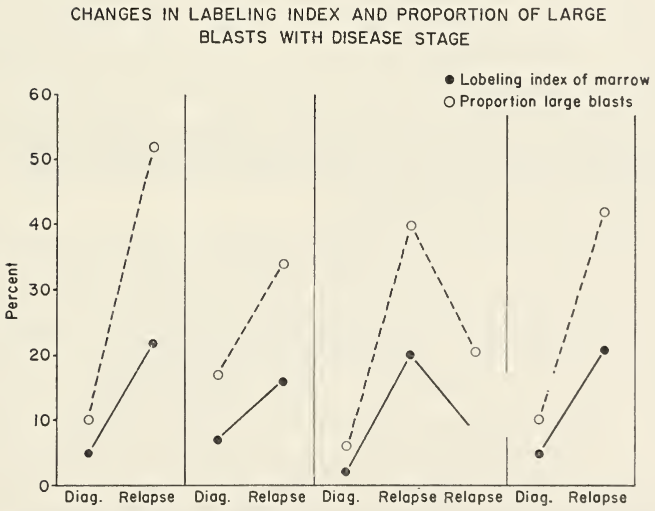
Three animal models in which a similar variable growth fraction has been demonstrated are the mouse breast tumor [Mendelsohn (*15, 16*)], the mouse ascites tumor [Baserga (*17*)], and the mouse fibrosarcoma [Frindel and co-workers (*18*)]. Frindel and co-workers demonstrated not only progressive accumulation of nonproliferating cells but also that during this time there was no change in the generation time of dividing cells. Therefore, changes in labeling index were indeed related to a progressive accumulation of nonproliferating cells.

Another feature of the leukemic cell population is concerned with appearance and turnover in blood. Cells in the blood are progressively replaced by cells coming from the marrow (text-fig. 14). The appearance of labeled cells in these two studies indicated that blood leukemic cell turnover occurred at about 10–15% per day.

The character of the leukemic cell in the blood is unknown. Do the leukemic cells in blood turn over until death like normal neutrophils, or do they recycle like lymphocytes? More importantly, what is the nature of the small leukemic cell? Is the small leukemic cell a nonproliferating end-stage cell like the neutrophil or is it more like the lymphocyte, a cell currently resting but which has the potential for reentering a phase of cell proliferation?

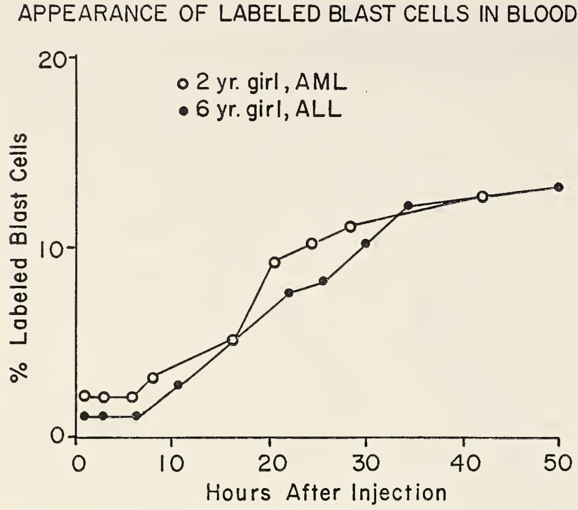


TEXT-FIGURE 12.—Changes in labeling indexes in 10 patients at diagnosis and in relapse.

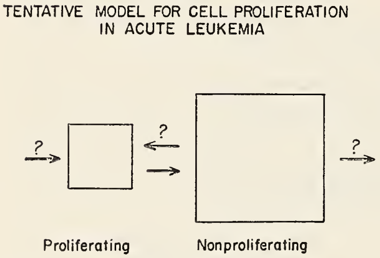


TEXT-FIGURE 13.—Comparison of labeling indexes and proportion of large cells in 4 patients at diagnosis and in relapse.

Text-figure 15 summarizes where we are today as far as cell kinetics in acute lymphocytic leukemia are concerned. In childhood leukemia there is a proliferative compartment within the marrow which is morphologically identifiable. The generation time for these proliferating cells is either normal or prolonged as compared to normal marrow elements. There are



Text-figure 14.—Time course for labeled leukemic cells in blood.



Text-figure 15.—A simplified schematic model for the leukemic cell population.

no data available on generation times for normal lymphocytes in man. In the proliferative compartment, these cells, after one or more divisions, become small and nonproliferating.

The unsolved questions are marked by the arrows in text-figure 15, indicating significance of the nonproliferative compartment. We have no information on rates of cell death nor on survival time of these small cells once they become small. Gavosto and his co-workers (*19*) recently raised a question about the proliferative compartment. In computing production rates of proliferative cells and their rate of appearance into the nonproliferative compartment, they observed what they believe to be a discrepancy; that is, more large cells become small than are replaced by rebirth within the proliferative compartment. Therefore, if there is no true stem cell compartment that is self-replacing, where do the leukemic cells come from? Do they result from a continued transformation of normal cells to leukemic cells or is this replacement by reentry of nonproliferative cells into a phase of proliferation? The latter concept is most important to our studies of therapy for this disease, as demonstrated recently by Miller and Cole (*20*) who studied the resistance of nonproliferative lymphocytes in rats to therapy with prednisone, cyclophosphamide, 6-mercaptopurine, and actinomycin D. These authors were primarily concerned with the persistence of immunologically competent cells, and much of their work is relevant to acute leukemia. They found these cells to be very resistant and to persist after rigorous therapy. They also found that these cells were available at some future time for reentry into a cycle of division.

We have no data yet to indicate that the nonproliferating cells of human acute lymphocytic leukemia return to a phase of division. However, there are some animal models which suggest that nonproliferating cells reenter a phase of division. For instance, Baserga and Gold (*21*), using Ehrlich ascites tumor cells, demonstrated that nonproliferating ascites tumors implanted into a new host would immediately reenter a phase of DNA synthesis and subsequent division. Frindel and his co-workers (*18*) likewise suggested this possibility in their tumor model. If this is so in human leukemia, then I think this nonproliferating, relatively chemotherapy-resistant tumor population may very well be the most important population we have to deal with in terms of therapy.

To go from this point and finish on perhaps more speculation than we can really support, maybe we ought to think more in terms of ascertaining what determines the relationship between dividing and nondividing cells and not so much of attempting to destroy this population. Perhaps if the population could be converted to a nonproliferating compartment entirely, it would be a perfectly supportable population as far as the host is concerned. Maybe we ought to think in terms of getting these nonproliferative cells back into a phase of cell division and perhaps even synchronizing them and making them particularly susceptible to intensive chemotherapy. These are some of the points in response to Dr. Cronkite's question: "Have studies of cell kinetics taught us anything yet?"

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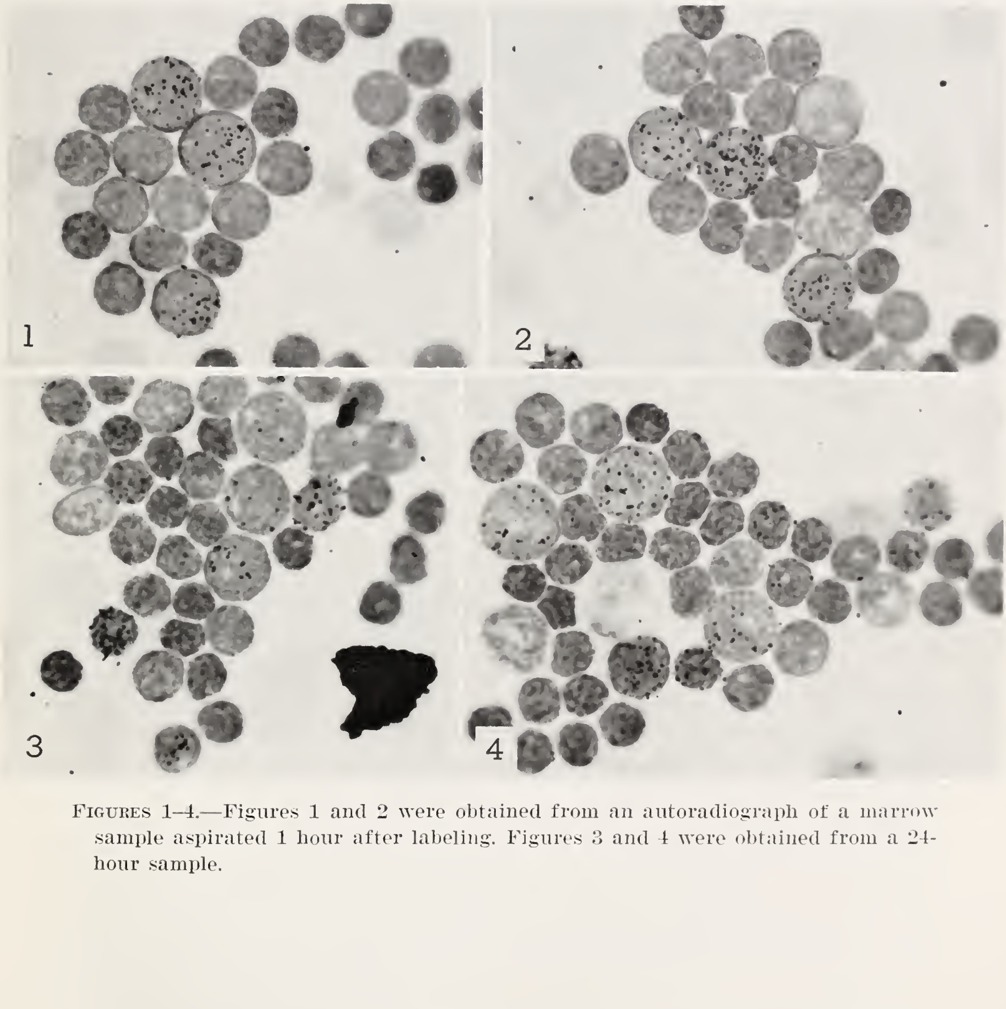

FIGURES 1–4.—Figures 1 and 2 were obtained from an autoradiograph of a marrow sample aspirated 1 hour after labeling. Figures 3 and 4 were obtained from a 24-hour sample.

Review of Recent Studies of Cellular Proliferation in Acute Leukemia[1]

Bayard D. Clarkson,[2] *Division of Chemotherapy Research, Sloan-Kettering Institute for Cancer Research, and the Department of Medicine, Memorial and James Ewing Hospitals for Cancer and Allied Diseases and Cornell University Medical College, New York, New York 10021*

I shall describe some of the studies we have been doing to understand the proliferative behavior of acute leukemic cells. Many uncertainties remain, but some things are becoming clearer and I think the overall pattern is emerging. We feel that the kinetics of proliferation in human leukemia are quite different from those in transplanted murine leukemia, in which most of the cells proliferate exponentially. Therapeutic principles arrived at from mouse leukemia models may not necessarily be directly applicable to human leukemia. Therefore it is important to define as precisely as possible the proliferative behavior of leukemic cells in different patients during different stages of the disease and also to investigate the interrelationship between leukemic and normal hematopoietic cells.

In most of our studies either single injections or continuous infusions of ^{3}H-thymidine (^{3}H-TDR) have been given, and autoradiographic methods and grain-count analysis have been employed as previously described (*1–4*). Because of the complexity of the system, I will discuss several different aspects of leukemic cell proliferation more or less separately; but as they are clearly interrelated, I will try to relate each to the overall proliferative behavior of the disease as we presently see it. Some of my conclusions are only tentative or even conjectural since they are based on limited experimental data, and may need to be modified as more complete information becomes available.

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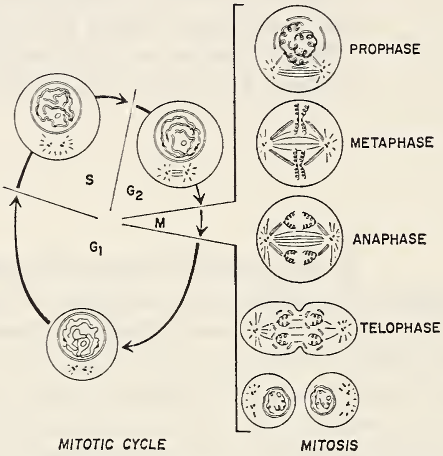
DURATION OF PHASES OF MITOTIC CYCLE IN LEUKEMIC CELLS

The classic picture of the mitotic cycle is shown in text-figure 1: This consists of a short mitotic (M) phase subdivided into its long-recognized different stages of mitosis; a postmitotic (G_1) phase of variable duration; a DNA synthetic (S) phase; and then a relatively short premitotic (G_2) phase, during which the cell prepares to undergo division. The major events that take place during each phase are described elsewhere (*5*).



TEXT-FIGURE 1.—Phases of mitotic cycle. M=mitotic; G_1=postmitotic; S=DNA synthesis; and G_2=premitotic.

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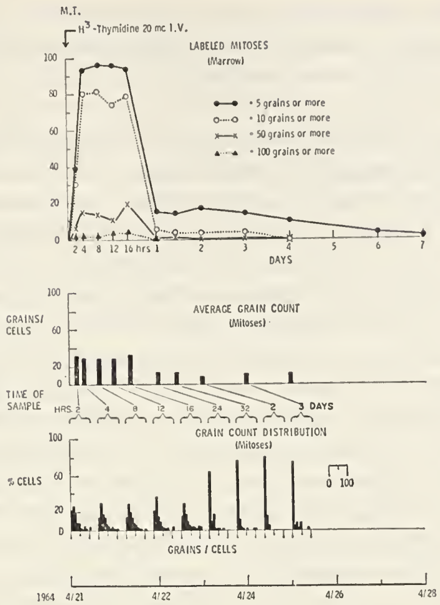
One way to estimate the durations of the phases of the mitotic cycle is to determine the time of appearance of labeled cells in mitosis after administration of a single injection of ^{3}H-TDR; under the experimental conditions used, this isotope is incorporated only by cells engaged in DNA replication (S phase). ^{3}H-TDR is degraded rapidly in the body and is available for incorporation by the cells for less than an hour (*6*).

One such study in a patient with acute myelomonoblastic leukemia is shown in text-figure 2 (*1*). Labeled mitoses have already begun to appear 2 hours after injection, and they reach a near maximum after about 4 hours. There is then a plateau, which lasts for about 12 hours, during which cells which had been in an earlier stage of DNA synthesis at the time of ^{3}H-TDR administration continue to progress through S and G_2 and reach mitosis. After most of this cohort of labeled cells, *i.e.*, those that were in the S phase when ^{3}H-TDR was injected, have completed division, the percent of labeled mitoses falls rapidly as (unlabeled) cells, in G_1 when the isotope was given, begin to divide.



Text-figure 2.—Time of appearance of labeled marrow leukemic cells in mitosis in patient M.T. with acute myelomonocytic leukemia following a single injection of ^{3}H-TDR. For the grain-count distributions, the grain counts are plotted in 11 groups *left to right:* 5–9, 10–19, 20–29, up to 90–99. Cells with over 100 grains are grouped on the *right.*

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The mean duration of S is measured as the time between the midpoints of the rising and falling curves of percent labeled mitoses—in this case about 19 hours (*1*). The median duration of G_2 equals the time between injection of ^{3}H-TDR and labeling of 50% of mitoses. Here it is about 3 hours.

We ourselves have studied only two patients with acute leukemia by means of labeled mitosis curves, chiefly because patients are reluctant to agree to enough marrow aspirates to carry out this type of study. However, a few other investigators have now performed labeled mitosis studies in several other patients with acute leukemia, and in all these published reports the mean duration of S in the leukemic cells was approximately 20 hours or slightly less (*7–9*).

The shape of the curve in text-figure 2 indicates that the average duration of S is reasonably constant in most leukemic cells. If the extent of ^{3}H-TDR incorporation is accepted as a reasonably valid measure of the rate of DNA synthesis (*10*), then the observation that the mean grain count remains almost constant during the first wave of mitoses indicates that the overall average rate of DNA synthesis is also fairly constant during most of the S phase.

The grain-count distribution data in text-figure 2 show considerable variability in the grain counts of the mitoses at each period during the first wave. If everything were constant in all cells, *i.e.*, rate of DNA synthesis, extent of utilization of ^{3}H-TDR, duration of G_2, etc., then at any one time during their first division all the mitotic cells should presumably have been in approximately the same stage of S when ^{3}H-TDR was injected and one might expect that their grain counts would be about the same. However, this was not the case, and the variability of their labeling intensity suggests that individual cells might have significantly different rates of DNA synthesis. It should be stressed that the method reflects only the average behavior and does not allow accurate determination of the variability in either rate or duration of S among individual cells.

Hale and Cooper (*11*), using combined autoradiographic methods and quantitative cytochemical measurements of DNA content, reported that individual leukemic cells may vary in their rate of DNA synthesis. Significant differences have also been reported in the rate of ^{3}H-TDR incorporation among different pairs or groups of human leukemic chromosomes and also between different segments of individual chromosomes (*12*).

Besides variability in synthetic rate, possible explanations for the variability in labeling intensity of the mitoses in our two patients include aneuploidy, differences in exposure to the isotope at different marrow sites, differing contents of thymidine kinase or pool sizes of endogenous thymidylic acid, and variable cell geometries with respect to uptake of isotope as well as to particle emission and contact with the autoradiographic plate (*1*, *3*). Although we did not determine the variability of these factors, I very much doubt there were sufficient differences in any of them to account for the large differences observed in labeling intensity.

At present I tend to favor the explanation proposed by Alpen and Johnston (*13*). These investigators, using ^{3}H-TDR, found that the mean DNA synthetic rate of nucleated erythroid cells in the dog was fairly constant, except at the beginning and end of the S period when it was reduced. However, they observed considerable variability in the grain counts of individual cells at any given location in S. To explain these findings, they proposed that DNA synthesis is an intermittent process, that chromosome replication proceeds on a more or less random basis, and that the apparent synthetic rate for an individual cell reflects the number of chromosomes or synthetic subunits in the process of DNA synthesis at any given time.

In neither of our studies did we observe a clear second rising wave of labeling mitoses. Presumably this was either because we did not obtain enough samples or more probably because the leukemic cells were dividing very asynchronously. One puzzling feature in both studies was that, after completion of the first wave of labeled mitoses (about 1 day), some labeled mitoses persisted and these were almost all very lightly labeled (text-fig. 2). Several explanations have been proposed (*1*):

One possibility is the reutilization of ^{3}H-TDR, but we do not believe this significantly affected the results because the percent of labeled interphase cells did not increase simultaneously with the labeled mitoses (*1*).

A second possibility is that the persistent labeled mitoses after the first day were cells dividing a second time and that only lightly labeled ones were generally present because the more highly labeled cells were inhibited as a result of radiotoxicity, from undergoing a second division. However, this explanation also seems inadequate since we know from other studies that cells containing even higher concentration of ^{3}H-TDR than the hottest ones in these two patients are able to divide repeatedly.

A third possibility is that some of the lightly labeled mitoses were cells that had a very slow rate of DNA synthesis and a correspondingly long S phase. Such cells would presumably incorporate less ^{3}H-TDR than the majority of cells that had divided earlier. However, the steepness of the descending curve of percent labeled mitoses indicates that most of the cells had a fairly constant duration of S. To conform to this explanation, one would have to postulate the existence of two almost distinct subpopulations of cells, one with an S phase of 19 hours and the other with a much slower synthetic rate; this seems implausible.

A fourth possible explanation is based on an observation reported by Moffat and Pelc in investigation of regenerating cells in the growing hair follicle of the mouse (*14*). ^{3}H-TDR was injected at the time of plucking, and autoradiographs were prepared by the standard paraffin technique as well as by the cryostat technique, which retains soluble compounds. It was concluded that some cells, which had not yet begun to synthesize DNA, phosphorylated ^{3}H-TDR and retained it in an intranuclear thymidylic acid pool; later, when the cells started DNA synthesis, they utilized this stored (labeled) thymidylic acid. Retained thymidylic acid not incorporated into DNA is presumably largely washed out by the solvents used in conventional techniques for preparing autoradiographs such as we employed. I consider this fourth alternative to be the most likely explanation for the persistent lightly labeled mitosis after the first day.

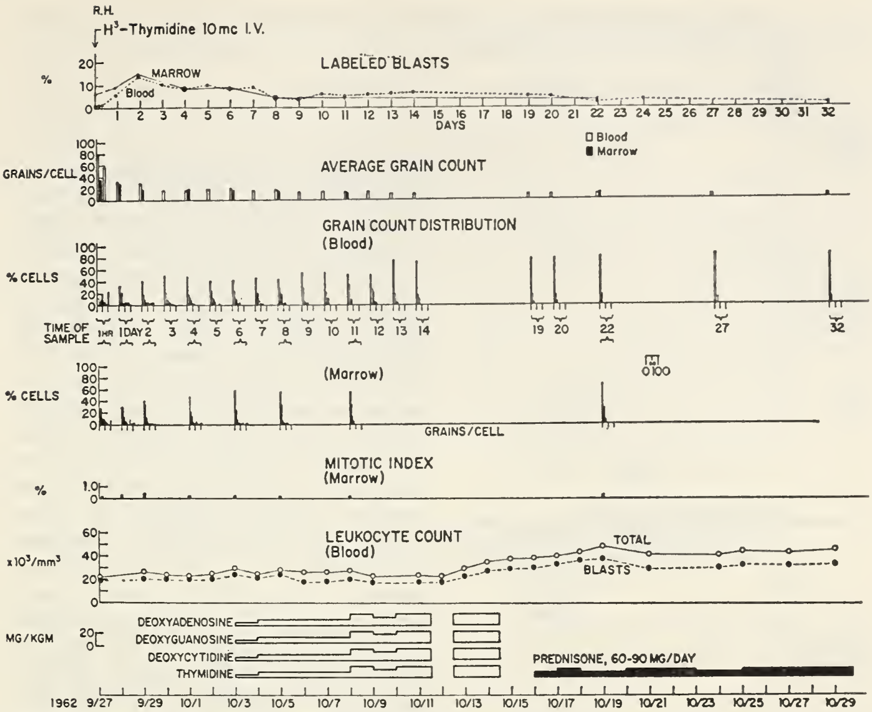
To summarize, the limited information thus far available (*1*, *7–9*) indicates that the average duration of S in human leukemic cells is usually about 20 hours and that the average rate of DNA synthesis is fairly constant during the greater part of the S period. However, it must be emphasized that all these estimates for the duration of S were arrived at in patients whose marrows were fairly densely populated with leukemic cells. As will be discussed, the cells may have a shorter synthetic period when they are less crowded, perhaps closer to the 13-hour estimate obtained by Stryckmans *et al.* (*15*) and the 16-hour estimate obtained by Todo (*9*) for normal granulocyte precursors. There have not been enough sufficiently detailed studies to make possible any firm conclusions regarding the extent of variability in either the duration of S among individual cells, or in the precise synthetic rates during all portions of S, or to know whether leukemic cells are more variable in these respects than are normal hematopoietic precursors. Differing results have been reported in various cell types growing *in vivo* or *in vitro* under different experimental conditions; in some, the rate of ^{3}H-TDR incorporation during DNA synthesis has been almost constant, whereas in others the cells have shown nonuniformity in rate of labeling (*13*, *16–19*).

LABELING OF INTERPHASE LEUKEMIC CELLS AND RATE OF RELEASE FROM MARROW TO BLOOD

In the marrow, the percentage of leukemic cells engaged in DNA replication at any one time varies rather widely in different patients, as demonstrated either by single injections of ^{3}H-TDR *in vivo* or by short-term incubation *in vitro*. In most studies of a series of patients, the average initial ^{3}H-TDR labeling index of marrow leukemic cells was around 6–10% (*1*, *8*, *20–25*), but in individual patients it varied from 0.2%, or less, to as high as 63% (*8*, *20*, *24*). As will be discussed, treatment may affect the percentage of leukemic cells synthesizing DNA, and several investigators have found that the ^{3}H-TDR labeling index may sometimes be higher during relapse than at the time of initial diagnosis (*2*, *3*, *8*, *24–26*). The initial labeling index of the circulating leukemic cells is almost always lower than the index of those in the marrow (*1*, *22–24*, *27*), although we have seen in a few patients with very large numbers of circulating leukemic cells a labeling index that was almost the same in the blood as in the marrow (*2*).

A typical example of the labeling pattern of leukemic cells in the marrow and blood after a single injection of ^{3}H-TDR is shown in text-figure 3. The labeling index of the marrow cells usually increases during the first day as the labeled cells divide, and then falls as unlabeled cells (which were in G_1 at the time of the ^{3}H-TDR injection) begin to divide. The labeling index then gradually decreases as the labeled cells undergo repeated divisions and the grain counts of the less highly labeled ones fall below the counting threshold. The labeled cells in the marrow rapidly pour out into the circulation and usually there is equilibration between the marrow and the blood by the end of the second day, the exact time depending on the rapidity with which the cells are released from the marrow in different patients.

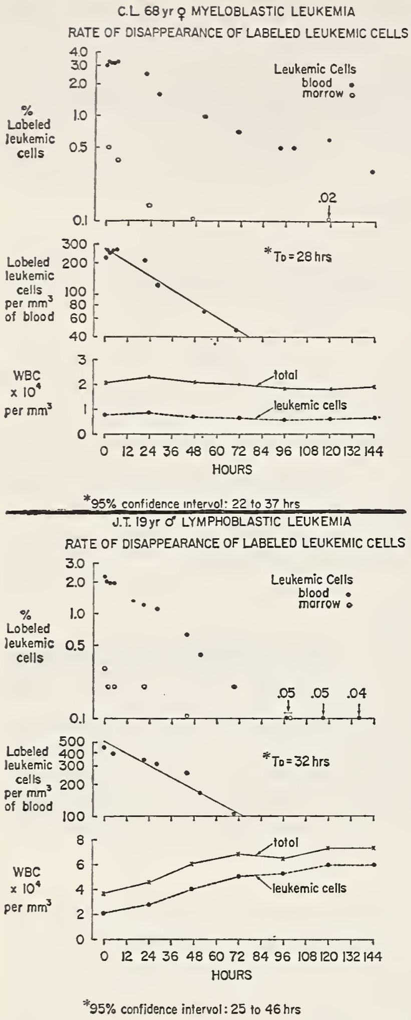
The leukemic cells do not divide in the circulation in most patients, although we have seen a few mitoses in the blood in some patients with very high white blood cell counts. The cells are apparently preferentially discharged from the marrow following division, *i.e.*, in G_1 rather than in S, since their labeling index in the blood is generally lower than in the marrow, but because they have a limited lifespan in the circulation, the conditions in the blood usually reflect those in the marrow with a time lag of about a day or two.



Text-figure 3.—Labeling pattern of interphase leukemic cells in marrow and blood of patient R.H. with acute myeloblastic leukemia after a single injection of ³H-TDR. The purine and pyrimidine deoxyribonucleosides were given by continuous infusion, as indicated, as part of an experimental program to test their possible therapeutic effect; there was no evidence that these compounds significantly altered the course of the disease.

We believe that, of the leukemic cells that enter the blood, most are destined to die, although obviously not all do so, since they sometimes multiply in extramedullary sites. Killmann and his associates (*28*) and ourselves (*1*) have determined the rate of disappearance of the most highly labeled blasts in four patients, starting after they reached a peak in the blood following single injections of ³H-TDR. In our two studies the half time of disappearance was around 25 hours and the upper limit of the mean transit time, *i.e.*, the average time spent in the blood, was about 36 hours; Killmann found a maximal mean transit time of about 33 hours.

To confirm these estimates we have done several studies in which we have taken about 500 ml of blood from patients with many circulating leukemic cells, labeled the cells *in vitro* for a short time with ³H-uridine, and then reinfused the autologous cells into the patient, using methods that will damage the cells as little as possible. The results of two such studies are shown in text-figure 4. Under the experimental conditions, almost all leukemic cells were labeled, but if the absolute number of labeled cells infused is compared with the number one would expect to



Text-figure 4.—Disappearance rate of ^{3}H-uridine-labeled leukemic cells from blood in 2 patients with acute leukemia. In these studies approximately 550 ml of blood was withdrawn into a 500 ml Fenwal Blood-Pack Unit. ^{3}H-uridine of specific activity 8 c/mM (1 mc in C.L. and 3 mc in J.T.) diluted in 10 ml of saline was injected into the bag and mixed thoroughly, and the unit was then incubated at 37°C for 2 hours. A 10,000-fold excess of nonradioactive uridine was then added and the blood was reinfused rapidly into the patients, the exact amount being determined by weighing. About 99% of the leukemic cells were labeled ($>$5 grains) *in vitro* in both studies. C.L. received 4.3×10^9 labeled leukemic cells and J.T., 11.2×10^9. (Estimated blood volume in C.L. = 3800 ml and in J.T. = 4200 ml.) The autoradiographs were exposed for 8 weeks in C.L. and 4 weeks in J.T. The average grain counts of the labeled cells fell from initial levels of 75 to 46 grains per cell at 142 hours in C.L., and from 87 to 30 grains per cell at 136 hours in J.T.

find in the circulatory compartment immediately after reinfusion, only about one-quarter to one-fifth of the anticipated number can be accounted for. Although rapid cell death cannot be excluded, it seems unlikely that so many cells that are metabolically active with respect to RNA synthesis would die so quickly. The discrepancy is more likely due to loss of cells into tissues or to their margination on vessel walls in a manner analogous to that seen after reinfusion of normal granulocytes labeled with DFP^{32} (*29, 30*).

Note in text-figure 4 that about 2–3% of the leukemic cells in the blood are labeled immediately after reinfusion but only about 0.3–0.5% in the marrow. The marrow samples were obtained by aspiration, and the small number labeled in the marrow are probably mostly cells circulating though the marrow sinusoids rather than cells that were returning to the marrow parenchyma to resume proliferation. In any case, there is no evidence of concentration of labeled cells in the marrow with time. The half times of disappearance in these studies were 28 and 32 hours.

Another reason to believe that many leukemic cells die is that calculations based on estimates of generation time and size of the proliferating fraction indicate that many more cells are being produced than can be accounted for, and that therefore there must be a substantial death rate (*1*, *28*, *31*). Possibly, some leukemic cells die *in situ* in the marrow, but there are no accurate techniques to measure this. However, from the autotransfusion experiments, it appears that most cells entering the blood have a limited lifespan and do not resume proliferation.

MATURATION OF LEUKEMIC CELLS IN ACUTE LEUKEMIA

It is generally agreed that the essential defect in acute leukemia is a failure of the leukemic cells to differentiate so that they persist as primitive cells that continue to proliferate and accumulate in the marrow; the normal cells meanwhile continue to differentiate, and most of their progeny develop into mature functional cells and leave the marrow, as in the normal state (*1*, *4*). However, acute leukemic cells do not always show a total lack of maturation, and in fact it is their ability to develop some specialized features that permits diagnosis of the morphological type of leukemia, *e.g.*, Auer bodies, monocytoid configuration, granules, specific enzymes that can be identified histochemically, etc. Of the various forms of acute leukemia, we have found that maturation can be most clearly demonstrated in acute myelomonocytic or acute monocytic leukemia, since in this type of leukemia the leukemic cells usually have a sufficiently different morphological appearance to be distinguishable from normal hematopoietic cells (*2*).

Examples of the leukemic cell types in a patient with acute myelomonocytic leukemia are shown in figure 1. We have arbitrarily divided the cells into Types I, II, and III, based on increasing levels of maturity. Two Type I cells of different size are shown to illustrate that cell size and maturation are not necessarily related phenomena, as well be discussed. There is still defective and incomplete maturation, since the most differentiated leukemic cells that could be recognized (Type III) are still abnormal and not fully mature. However, they have matured to the extent of losing their proliferative potential. The Types I and II cells label immediately on flash exposure to ^{3}H-TDR, but the Type III cells generally do not (*2*); the latter are therefore in a stage of development analogous to that of normal metamyelocytes, which are also no longer capable of reproduction.

The Type I cell is the earliest form of leukemic cell we could recognize. Gavosto has suggested that the recognizable leukemic cells do not behave as a self-maintaining system, and that there must be an influx of leukemic "stem cells" from an unrecognized precursor compartment to perpetuate the disease (*32*). We cannot exclude this possibility, but as discussed elsewhere (*3*) all our data are consistent with the hypothesis that the recognizable leukemic cells do function as a self-maintaining population.

We have not done a systematic study to determine in what percentage of patients there exist leukemic cells that can mature sufficiently to lose their ability to reproduce, but I suspect that this occurs frequently, at least in acute myeloblastic, myelomonocytic, or monocytic leukemia. Before we began studying proliferation kinetics, I had been under the impression that leukemic cells behaved like normal cells in that the more mature cells had a higher expectancy of entering the blood than the less-differentiated ones. Like many hematologists, when, after examining the marrow, I am uncertain as to the morphologic type of leukemia, I examine the peripheral blood smear on the supposition that the cells in the blood often seem more mature. However, somewhat contrary to this impression is the observation that, in several patients we have studied carefully and in whom the leukemic cells could clearly be divided into three grades of maturity, the distribution of the three types was fairly similar in the marrow and blood (*2*).

Populations of leukemic cells which contain cells that can mature to the extent of becoming incapable of further division might be expected to expand more slowly than populations composed entirely of undifferentiated (Type I) cells. I am still not certain about this point, but we have studied a limited number of patients with different types of acute leukemia and have seen no clear correlation between the absence or presence of partially mature cells and the proliferative rate of the whole leukemic cell population. One of the most rapidly expanding leukemic cell populations we have studied contained definite Type III cells, although there were relatively few of them when the leukemia was in a rapid stage of expansion (*2*).

We have not been able to draw similar conclusions regarding maturation of leukemic cells in lymphoblastic leukemia. This failure has in part been due to the fact that it is usually impossible to distinguish between leukemic cells and normal immature lymphocytes. Moreover, although normal lymphocytes are usually thought to become smaller as they mature, at least some of these small lymphocytes have the capacity to undergo blastic transformation and resume proliferation (*e.g.*, after stimulation with phytohemagglutinin), and their behavior is therefore not analogous to that of granulocytes.

RELATION OF CELL SIZE TO MATURATION AND PROLIFERATIVE ACTIVITY

Although changes in cell mass and maturation may be related phenomena, to understand the kinetics of leukemic cell proliferation we should regard them as independent processes. This is emphasized because the two processes have been confused in the literature.

Gavosto and his associates (*33*) first reported that large leukemic cells have a much higher initial ^{3}H-TDR labeling index than small ones. This observation has subsequently been confirmed in several laboratories (*7*, *8*, *24*) including our own (*2*, *3*). Because Gavosto and his co-workers found that labeled and unlabeled blasts in normal bone marrow did not vary as much in size as did leukemic blasts, they concluded that cellular growth preparatory to division had no significant influence on their finding that the larger leukemic blasts showed greater DNA synthetic activity. They further concluded that acute leukemic cells have an absolute incapacity for differentiation and maturation, and that as the cells age without maturing they become smaller and lose the ability to divide.

Whereas leukemic cells in some patients are morphologically homogeneous and show little sign of maturation, some leukemic cells in other patients may undergo definite maturation, sometimes to the extent of losing proliferative potential. Moreover, at least in acute myelomonocytic leukemia, the cells may become larger instead of smaller as they mature (*2*).

We have shown, by serial sampling in several patients after single injections of ^{3}H-TDR, that the smaller leukemic cells became labeled as a consequence of the larger ones halving their volume by division (*2*). Some of the small cells then enlarge and divide again; it is apparently necessary that the cells achieve a certain sufficient mass before they can resume DNA synthesis and reenter the division cycle. The process of cell growth is separate from that of maturation, but it may take place more or less simultaneously in some cells. All our data are in accord with a heterogeneous size distribution of the leukemic cells and continuous interconversion between cells of different sizes as a result of cellular growth followed by halving of volume by division (*2*, *3*).

SIZE OF PROLIFERATING FRACTIONS OF LEUKEMIC POPULATIONS

During the last few years, a number of reports with varying degrees of emphasis have introduced the concept that there is a proliferating pool composed of large blasts and a more or less distinct nonproliferating pool composed of small blasts within a given population of leukemic cells (*21*, *31–36*). Mauer and his co-workers originally concluded that the proliferating pool might be as small as 12% in some patients (*34*). We do not believe that the evidence presented for such a large nondividing fraction is convincing, and in none of the studies on which these conclusions were based was the possibility excluded that the small, so-called nonproliferating, cells might later begin to divide.

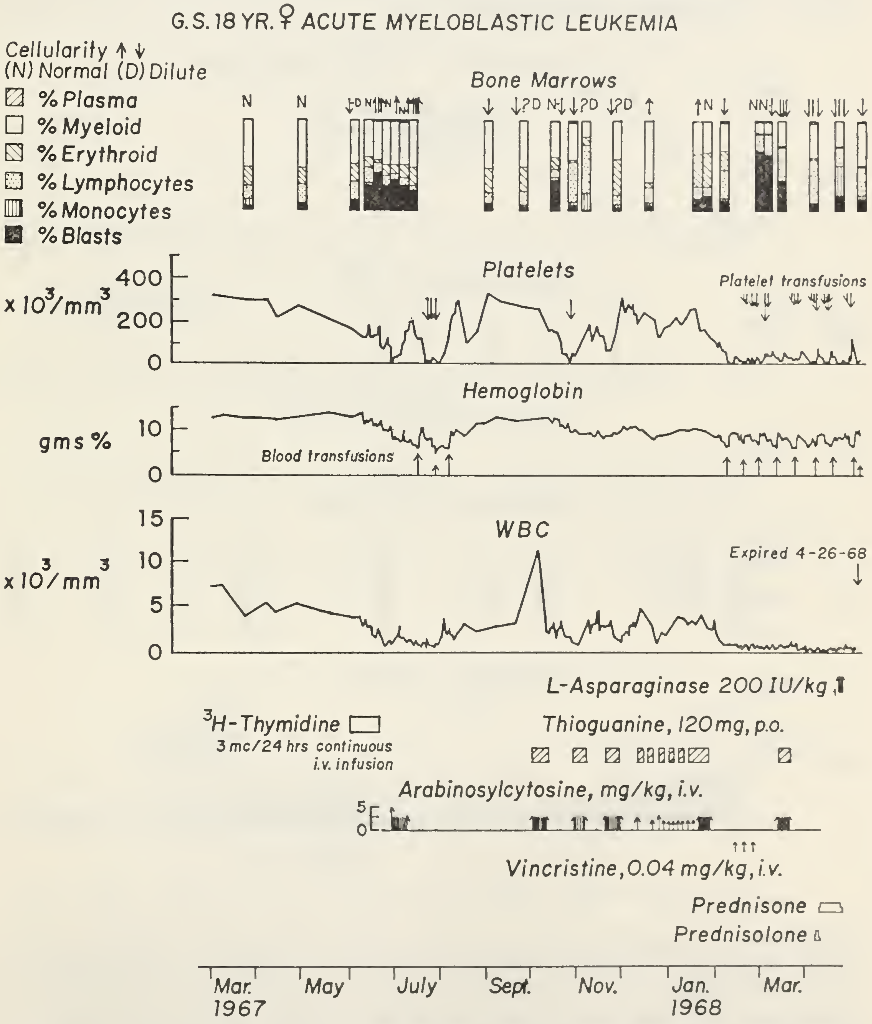
Our own studies (*3*, *37–39*) demonstrate that in most patients the majority of leukemic cells proliferate, but their generation times vary widely (mostly due to variation in G$_1$). This is not to deny the importance of the relatively small fraction of cells that divide very slowly or may remain dormant for long periods, since it may well be that these cells escape the action of current chemotherapeutic regimens and are eventually responsible for relapse of the disease. However, we believe these dormant cells do not constitute a separate or unique subpopulation but merely represent one end of the spectrum in a population whose generation time is extremely variable. Moreover, although this has not yet been proved, we suspect that the dormant state is only temporary and that, when the condition of the disease changes, such dormant cells and their progeny may be able to resume proliferation at about the same average rate as that of the original whole population (*3*).

The best method to estimate the size of the proliferating fraction is to expose the cells continuously to ^{3}H-TDR, which, providing the concentration is sufficient, will be incorporated by, and will adequately label, all cells entering DNA synthesis (which then presumaby go on to divide) (*3*). If the experimental conditions are appropriate, *e.g.*, dosage of ^{3}H-TDR, auto-excluded that are not actually in S phase but which may incorporate very small amounts of ^{3}H-TDR, *i.e.*, as a result of DNA turnover or DNA repair in damaged cells (*40*).

We have now given continuous intravenous infusions of ^{3}H-TDR to 8 patients with acute leukemia, 5 of them for 8–10 days and 3 for 20–21 days. The conditions of these experiments were such that all cells entering the S phase were labeled, since we invariably found all blast mitoses to be labeled after the first few days. In 4 of the patients given infusions for 8–10 days, 7–12% of the leukemic cells in the marrow were still unlabeled at the end of the infusion; these unlabeled cells had therefore presumably been resting or dormant during this entire period (*3*, *39*). The fifth patient (*2*) showed a larger number of dormant cells as will be discussed later. In the 3 patients given infusions of 20–21 days' duration, 1, 2, and 8% of the leukemic cells in the marrow remained unlabeled at the end of the infusions (*38*, *41*). In all 3 of the latter patients the disease appeared to be progressing slowly from a clinical standpoint; one of them was untreated, whereas 2 had previously received treatment, although not for several weeks or months.

To illustrate one of these continuous infusion experiments, I will describe the results in one patient (G.S.) who was given a 20-day infusion. This patient, an 18-year-old girl, had acute myeloblastic leukemia whose disease progressed rather slowly according to usual clinical criteria. She had a complete remission following her initial treatment in September 1965, with arabinosyl cytosine which lasted for over 10 months without any maintenance chemotherapy before relapse was detected in the marrow (she refused to continue taking this drug as maintenance treatment because it caused severe vomiting). Complete remission was reinduced with arabinosyl cytosine during November and December 1966, but on this occasion it lasted only about 5 months before marrow relapse occurred (May 1967). Her subsequent course is illustrated in text-figure 5.

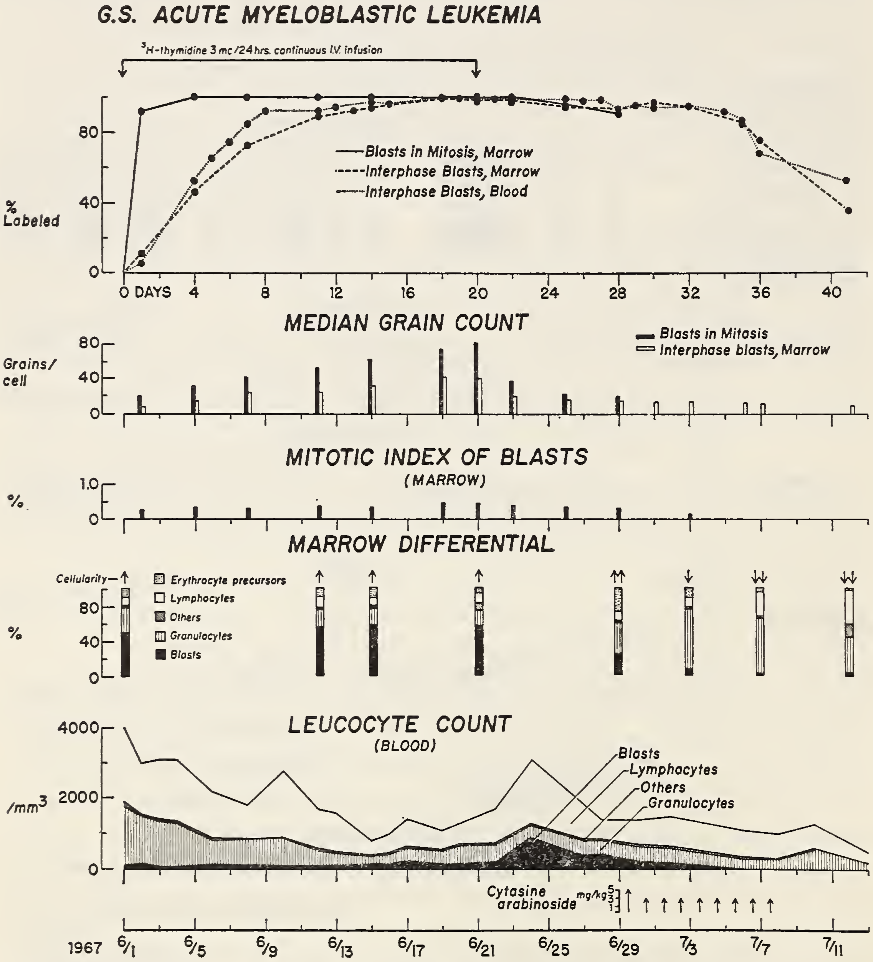
Most leukemic cells in this patient were primitive blast forms (Type I), but at different times during her course some of them developed large azurophilic granules and therefore should probably properly be classified as promyelocytes. Since these cells otherwise closely resembled the blasts



TEXT-FIGURE 5.—Clinical course of patient G.S. The ³H-thymidine study was done in **June 1967.**

and were almost certainly leukemic, and since their proportion varied, they were classified together with the primitive blasts. On several occasions during her course some of the myelocytes also appeared abnormal and were probably leukemic; however, since these abnormalities may sometimes have been related to therapy and since we could not always distinguish leukemic myelocytes from normal ones, none of the myelocytes were included with the leukemic cells.

The continuous infusion of ^{3}H-TDR was started on 6/1/67 during the patient's second relapse (text-fig. 5). The marrow on this date had 51%

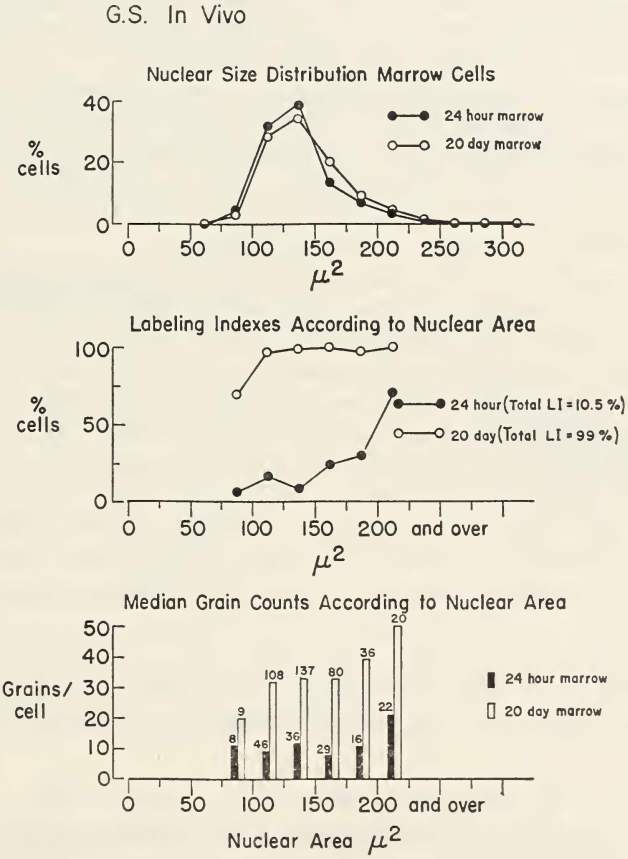


TEXT-FIGURE 6.—Labeling pattern of leukemic cells in marrow and blood of patient G.S. during and following continuous intravenous infusion of ^{3}H-TDR for 20 days. The median grain counts of the leukemic cells in the blood are not shown but were very similar to those of the interphase blasts in the marrow. Too few leukemic cells were found after 7/3/67 to determine their mitotic index, but none were seen in mitosis. The methods used are described in detail elsewhere (*3*).

leukemic cells, whereas the 3 most recent previous marrows on 3/13/67, 4/17/67, and 5/22/67 had shown 3, 5.5, and 9.5% leukemic cells, respectively. The percentage of leukemic cells increased only slightly during the infusion (to 56% on 6/21/67).

The initial ^{3}H-TDR labeling index of the leukemic cells on 6/1/67 was 6.3% in the marrow and 0.5% in the blood (as determined by *in vitro* incubation of the cells with ^{3}H-TDR for 1 hour immediately prior to start of infusion). At the end of the infusion, 99% of the leukemic cells were labeled in the marrow and 99.8% of those in the blood (text-fig. 6).

Text-figure 7 shows the size distribution of the leukemic cells in the marrow and their labeling frequency and intensity according to nuclear area at the beginning (after 24 hours) and at the end of the infusion.



TEXT-FIGURE 7.—Relation of nuclear size of marrow leukemic cells in G.S. to ^{3}H-thymidine labeling frequency and intensity after 24 hours' and after 20 days' infusion of ^{3}H-thymidine *in vivo*. The areas of 500 nuclei were measured in each sample as described elsewhere (*3*). The number of labeled cells in each size category which were counted to determine their median grain counts are indicated above each column of grains per cell.

The nuclear size distribution remained fairly constant during the infusion. Relatively few small cells were labeled by 24 hours, but those that were had median grain counts resembling those of all except the very largest cells; in this instance some of the largest cells may already have divided by 24 hours. When samples were obtained earlier (after 4 hours) in other patients given continuous infusions, the labeling intensities of the medium-sized and large-sized cells were very similar *in vivo* (*3*), indicating that they had similar rates of DNA synthesis.

Almost all leukemic cells were labeled regardless of size at the end of the 20-day infusion in patient G.S., but the few cells remaining unlabeled were generally small ones. Of the patients we studied, G.S. had the fewest cells remaining unlabeled at the end of the infusion, but the findings were similar in all our other ^{3}H-TDR infusion studies in that it was generally the small cells that remained unlabeled (*2*, *3*, *39*). It thus seems to be generally true that the leukemic cells that remain dormant for relatively long periods are mostly small cells.

As in most other infusion studies, the large leukemic cells in G.S. generally had significantly higher labeling intensities at the end of the infusion, indicating that they had passed through more division cycles during the 20-day period than had the small ones (*3*). This is in keeping with the conclusion that the generation time of the leukemic cells in any one population is variable, as will be discussed.

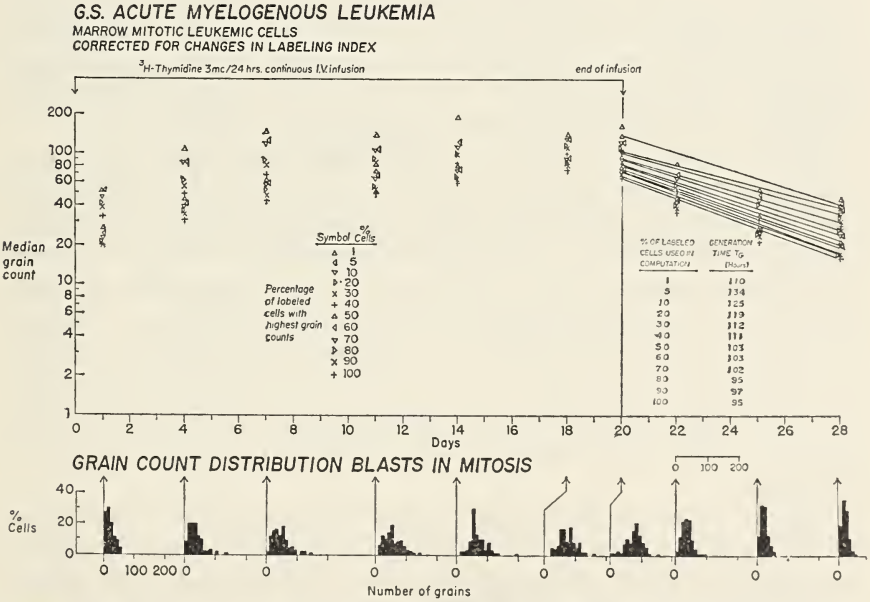
Since we have not done microdensitometric measurements of DNA content, we are not sure in what phase of the mitotic cycle the dormant cells are arrested, but probably most of them are in G_1. In all our infusion studies the unlabeled cells appeared to be viable, and in most cases in which we studied ^{3}H-uridine or ^{3}H-leucine incorporation (*37*), almost all cells, regardless of size, incorporated these isotopes (*e.g.*, see studies described in text-fig. 4), although the larger cells generally showed greater uptake (*3*). The so-called dormant cells are therefore usually not metabolically inert, and the designation "dormant" merely refers to their temporary cessation of active proliferation. We presume they are fully capable of resuming division later. If this is so, it seems more appropriate to say they are in G_1 rather than in G_0, but a more accurate delineation of this (presently semantic) distinction must await definition of the biochemical events which take place as a cell shifts from a truly resting state to one preparing to begin DNA synthesis and reentering the division cycle.

GENERATION TIME OF LEUKEMIC CELLS

There has been much controversy in the literature concerning the duration of the generation time of acute leukemic cells. Since this is very relevant to therapy, I will discuss this topic at length. The most accurate way to measure the generation time of the proliferating fraction of cells is to measure the time between corresponding points on consecutive rising waves of labeled mitosis. Of course, this method cannot be used if no second rising wave occurs, as in our two studies referred to earlier, *e.g.*, text-figure 2. Saunders, Lampkin, and Mauer (*8*) observed second waves of labeled leukemic cell mitoses in three patients, and in these studies the minimal generation time appeared to be about 60 hours.

We have used two independent methods to estimate the generation time (*1–3*, *38*); in both methods, only the actively dividing, *i.e.*, labeled cells are considered. These methods are relatively crude and the measurements are approximations, but we feel certain that they are of the correct order of magnitude. They have been developed in collaboration with Dr. Jerrold Fried, who has carried out all the computations and analyses. All determinations were performed with the aid of an IBM 1800 computer system. I will illustrate these methods by describing the results in patient G.S. referred to earlier (text-figs. 5–7), and then I will summarize the findings in other similar studies.

The first method, only applicable during continuous infusions of ^{3}H-TDR, involves just the leukemic cells in mitosis. It also allows a rough estimate of the variability of generation times among the dividing cells. The results of one such study in G.S. are shown in text-figure 8. This method



TEXT-FIGURE 8.—Computer curves of median grain counts of labeled leukemic cells in mitosis in the marrow of patient G.S. to illustrate methods used in determining their generation time during the infusion of ^{3}H-TDR and after it is stopped (*3*). No therapy was given until the 8th day after infusion ended. For the grain count distributions, the cells are plotted from *left* to *right* in 20 groups: 0–10, 11–20, and so forth, up to 190–200.

is based partly on the assumption that, at a suitable time after the infusion is started, all the mitotic cells will have traversed one complete period of DNA synthesis (S phase) after the body thymidine pool has reached a steady state with respect to specific activity of label. The grain count distribution of this sample of mitotic cells is then taken to represent the probability distribution for grains "taken up" by any cell during its passage through subsequent S phases during the infusion. By means of an assumed "trial" mean and variance of generation times, the grain count distribution of cells in mitosis can be predicted for times between the initial sample and the end of the infusion. Based on the difference between the resulting predicted distributions and those observed experimentally, a new trial mean and variance are postulated and the process is repeated.

Since most previous studies have shown that the average duration of the S phase in acute leukemic cells is about 20 hours (*1*, *7–9*), ideally a sample taken at about day 1 should be used to establish the grain-count probability distribution. However, the 24-hour sample in the present study proved unsatisfactory because: 1) Too few mitoses were present to establish a reliable grain count distribution (only 56 found on the entire slide), and 2) their labeling intensity was so low that a steady state could not yet have been reached. Therefore, the 4-day sample was used, even though it undoubtedly included some cells that had already traversed more than one S phase during the infusion. The labeling intensities of the mitoses increased gradually until the end of the infusion. In table 1 the observed and best predicted grain distributions are compared. In this case, the mean generation time was 95 hours; 67% of the cells had generation times between 50 and 185 hours, and 95% between 25 and 360 hours.

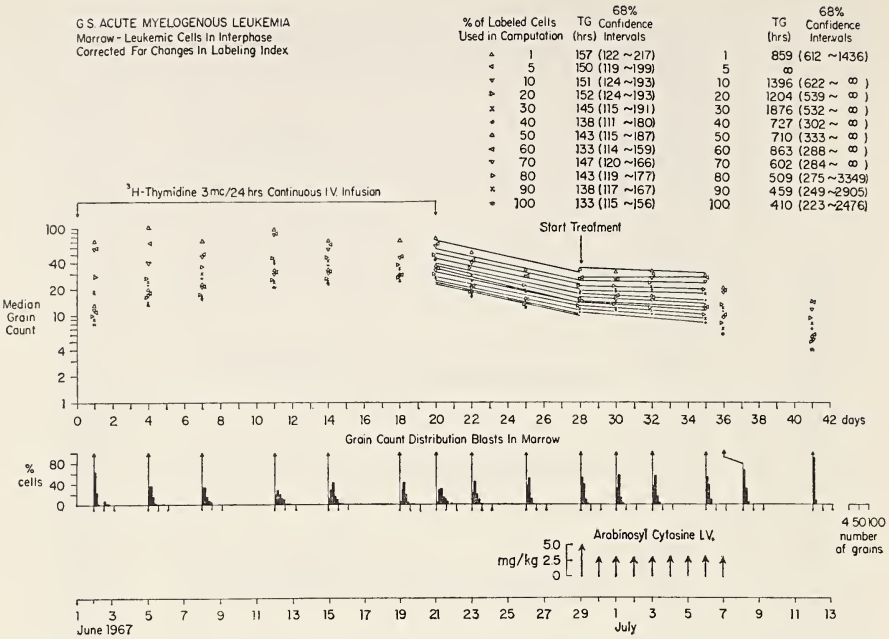
The other method, applicable to either single injections or continuous infusions of ^{3}H-TDR, is based on the time necessary for the median grain count of the labeled cells to halve after completing their first division following a single injection (*1*, *2*), or following multiple divisions at the end of a continuous infusion (*3*). In the marrow, both mitotic cells and interphase cells can be studied separately and the results compared. Although the leukemic cells rarely divide in the circulation because they generally have a relatively short transit time in the blood, the grain counts of the cells in the blood usually decrease at about the same rate as do those of the interphase cells in the marrow, and closely reflect the generation time of the latter.

Only the marrow leukemic cells in mitosis were considered in text-figure 8. The observed values for the median were adjusted to compensate for the changes in ^{3}H-TDR labeling index due to lightly labeled cells falling below the grain counting threshold after division (*1–3*). For reasons presented elsewhere (*3*), the generation time estimate generally tended to decrease as the percentage of labeled cells included in the computation increased until a plateau was reached (at about 95 hours); this value was taken as the best estimate of the mean generation time.

TABLE 1.—Comparison of observed and predicted distribution of grains in mitotic leukemic cells

Grain level	Range of grain counts	Time after start of ^{3}H-thymidine infusion (days)											
		1	4*	7		11		14		18		20	
		Observed (%)	Observed (%)	Predicted (%)	Observed (%)	Predicted (%)	Observed (%)	Predicted (%)	Observed (%)	Predicted (%)	Observed (%)	Predicted (%)	Observed (%)
1	0–10	27	8	3	2	1	0	0.4	0	0	0	0	1
2	11–20	30	19	9	12	5	8	2	2	1	0	0.7	1
3	21–30	20	20	14	15	10	12	7	2	5	5	4	3
4	31–40	11	20	18	16	15	10	12	3	10	4	10	1
5	41–50	9	12	16	12	17	14	16	9	15	9	15	7
6	51–60	4	10	14	17	16	19	17	30	17	19	17	11
7	61–70	0	2	9	9	12	7	14	14	16	15	16	8
8	71–80	0	3	6	4	8	8	11	10	12	9	13	16
9	81–90	0	3	4	5	5	10	7	10	8	15	9	21
10	91–100	0	0	2	3	4	5	5	4	5	9	6	13
11	101–110	0	2	2	0	3	4	3	8	4	6	4	8
12	111–120	0	0	1	2	2	0	2	3	2	4	3	5
13	121–130	0	0	0.6	2	1	1	1	1	2	3	2	1
14	131–140	0	0	0.2	0	0.5	0	0.8	1	1	2	1	1
15	141–150	0	1	0.4	0.9	0.3	2	0.4	0	0.5	0.7	0.5	0
16	151–160	0	0	0.3	0.9	0.3	0	0.3	0	0.3	0.7	0.3	0
17	161–170	0	0	0.2	0	0.3	0	0.3	0	0.3	0.7	0.3	1
18	171–180	0	0	0.1	0	0.1	0	0.2	0	0.3	0	0.3	0
19	181–190	0	0	0	0	0	0	0.1	0	0.1	0	0.1	0
20	191–200	0	0	0	0	0	0	0	1	0	0	0	0
Number labeled mitoses counted		56	156	101		100		96		151		75	

*4-Day sample was used to define probability distribution of grains taken up by cell in passing through subsequent S phases during remainder of infusion. Predicted values are based on assumed mean generation time of 95 hours, with 95% of cells having generation times between 25 and 360 hours (log normal distribution).



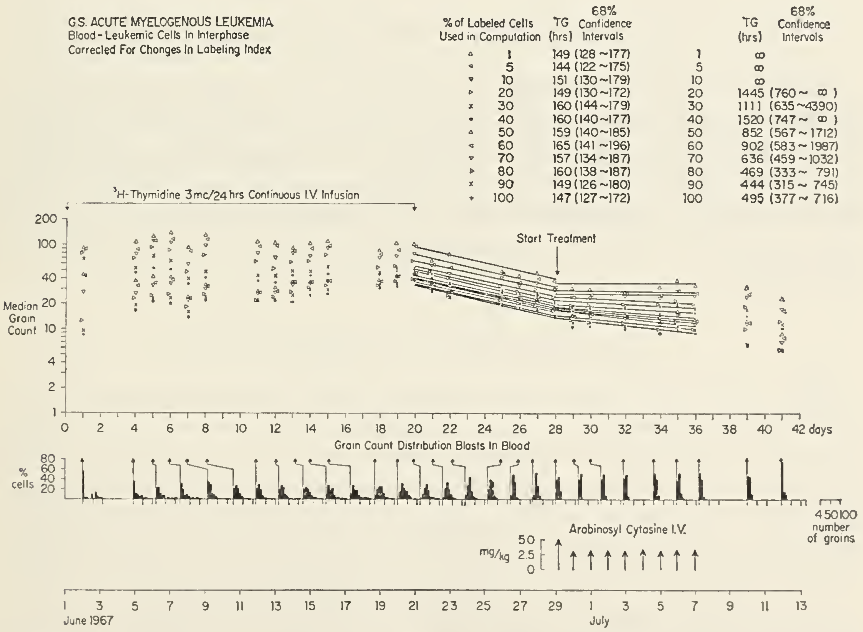
	% of Labeled Cells Used in Computation	TG (hrs)	68% Confidence Intervals		TG (hrs)	68% Confidence Intervals
▵	1	157	(122 ~217)	1	859	(612 ~1436)
◃	5	150	(119 ~199)	5	∞	
▿	10	151	(124 ~193)	10	1396	(622 ~ ∞)
▹	20	152	(124~193)	20	1204	(539 ~ ∞)
×	30	145	(115 ~191)	30	1876	(532 ~ ∞)
•	40	138	(111 ~180)	40	727	(302 ~ ∞)
▵	50	143	(115 ~187)	50	710	(333 ~ ∞)
◃	60	133	(114 ~159)	60	863	(288 ~ ∞)
▿	70	147	(120 ~166)	70	602	(284 ~ ∞)
▹	80	143	(119 ~177)	80	509	(275 ~3349)
×	90	138	(117 ~167)	90	459	(249~2905)
•	100	133	(115 ~156)	100	410	(223~2476)

TEXT-FIGURE 9.—Computer curves of median grain counts of interphase leukemic cells in marrow of patient G.S. illustrate method of determining their mean generation time at the end of the ³H-TDR infusion. The computation was performed separately both before and after treatment was started, and the change in slope of the curve is apparent. *See* text.

The same method was employed to estimate the mean generation time using interphase leukemic cells in the marrow (text-fig. 9) and blood (text-fig. 10) both before and after start of treatment; the effect of treatment will be discussed. Assuming that the estimates obtained with 100% of labeled cells are most accurate, mean generation time estimates of 133 hours and 147 hours were found for the marrow and blood interphase cells, respectively, between the end of the ³H-TDR infusion and the start of treatment. For confirmation, we repeated the measurements shown in text-figures 9 and 10, using the grain count data from another complete set of autoradiographs. Before treatment was started, estimates of 130 hours and 128 hours were obtained for the marrow and blood leukemic cells, respectively. The averages of these values were used as the best estimates of the mean generation of the interphase blasts: marrow = 131 hours, and blood = 138 hours.

In this instance, the generation time estimates found for the interphase cells were longer than for the mitotic cells. This difference probably indicates that some interphase cells entered a resting phase after passing through at least one division cycle, whereas the mitotic blasts were selected cells, in that they included few long-term resting cells. In other patients in whom the leukemic populations were expanding, we found the generation time estimates for the mitotic and interphase blasts to be almost the same (*3*, *41*). In such cases the mitotic blasts were more representative of the whole population, since few cells went into long resting periods after division.

Numerous patients with acute leukemia have now been studied with grain-count-halving methods similar to the second method described above. In untreated patients, or in those who were not receiving therapy during the study, the mean generation time estimates of the leukemic cells have almost always been between 2 and 8 days (*1–3*, *7*, *9*, *23*, *37*, *42*). Using different methods, Mauer and Fisher (*21*, *34*) concluded that only about 12% of the leukemic cells were proliferating in children with acute leukemia, and that the generation time of this proliferating fraction was about 15–20 hours. However, their evidence for such a short generation time was not convincing, and subsequently Saunders, Lampkin, and Mauer reported a generation time of about 60 hours in childhood leukemia (*8*). In most patients so far studied by means of labeled mitoses curves (*1*, *7–9*), the mean duration of S in leukemic cells has been found to be around 20 hours or only slightly less. Hence I find it hard to believe



% of Labeled Cells Used in Computation		TG (hrs)	68% Confidence Intervals		TG (hrs)	68% Confidence Intervals
△	1	149	(128 ~ 177)	1	∞	
◁	5	144	(122 ~ 175)	5	∞	
▽	10	151	(130 ~ 179)	10	∞	
▷	20	149	(130 ~ 172)	20	1445	(760 ~ ∞)
×	30	160	(144 ~ 179)	30	1111	(635 ~ 4390)
•	40	160	(140 ~ 177)	40	1520	(747 ~ ∞)
△	50	159	(140 ~ 185)	50	852	(567 ~ 1712)
◁	60	165	(141 ~ 196)	60	902	(583 ~ 1987)
▽	70	157	(134 ~ 187)	70	636	(459 ~ 1032)
▷	80	160	(138 ~ 187)	80	469	(333 ~ 791)
×	90	149	(126 ~ 180)	90	444	(315 ~ 745)
+	100	147	(127 ~ 172)	100	495	(377 ~ 716)

TEXT-FIGURE 10.—Computer curves of median grain counts of interphase leukemic cells in blood of patient G.S. illustrate method of determining their mean generation time at the end of the ^{3}H-TDR infusion. The computation was performed separately both before and after treatment was started, and the change in slope of the curve is apparent.

that the *average* generation time of the proliferating cells is shorter than 20 hours in any patient.

We have always found considerable variability in the generation times of the leukemic cells in any one patient (*1–3*, *37*). The extremes are not yet known, since our methods do not permit us to separately make an accurate estimate of the generation time of the fastest dividing cells, nor do they include the dormant cells, *i.e.*, those unlabeled at the end of the continuous ^{3}H-TDR infusions, which may go for long periods without dividing. However, judging from the rate of increase in labeling intensity of the largest blasts during continuous infusion studies (*3*), I estimate that some cells may divide as frequently as every 24 hours or slightly less. Judging from the infusion studies, I am almost certain that, at least in some patients with slowly progressive disease, some leukemic cells persist in the marrow for at least 20 or 21 days without dividing.

The rate of proliferation may vary considerably in different patients, and in any given patient it may be altered by therapy and vary according to the stage of the disease. As will be shown, not only may the mean generation time of the actively proliferating (*i.e.*, labeled) cells be affected but the size of the actively proliferating fraction may change.

EFFECT OF TREATMENT ON PROLIFERATION RATE

There have been surprisingly few studies on the effects of treatment on the kinetics of human leukemic cell proliferation. In most of our own studies, we used antimetabolites. We do not have enough information yet to relate any particular effects to the different mechanisms of action of the various types of drugs used in treating leukemia. I will first discuss the known immediate effects of chemotherapy and then discuss possible delayed effects.

Immediate Effects of Chemotherapy

When generation times were estimated by the grain-count-halving method, both immediately before and during treatment of patients with cytotoxic drugs, they were almost always found to be prolonged during treatment (*2*, *3*). Presumably, this is a consequence of either selective killing of the more rapidly dividing cells or of sublethal injury of other cells, which slows their passage through the mitotic cycle; we cannot distinguish between these possibilities by the grain-count-halving method.

An example of one such study in G.S. is shown in text-figures 9 and 10. After starting treatment with arabinosyl cytosine on the 9th day after the end of the ^{3}H-TDR infusion, there is a distinct change in the slopes of the median grain-count curves of both the marrow and blood interphase leukemic cells. The mean generation time estimates increased from 133 hours before treatment to 410 hours during treatment for the interphase marrow cells (text-fig. 9) and from 147 to 495 hours for the interphase blood cells (text-fig. 10). The mitotic index of the marrow leukemic cells fell to about a third of the pretreatment level after 4 days of therapy (text-fig. 6); no leukemic cells were seen in mitosis after this, although there were too few remaining for an accurate measurement of the mitotic index. After nine consecutive daily injections of arabinosyl cytosine, therapy was stopped because of thrombocytopenia (text-fig. 5).

Although the median grain counts decreased only slightly during treatment, the grain counts of the surviving leukemic cells began to decrease again as soon as treatment was stopped (text-figs. 9 and 10). There were too few leukemic cells remaining thereafter for a reliable estimate of the generation time of these residual cells. Note in text-figure 6 that the ^{3}H-TDR labeling index remained almost constant during treatment, but began to drop rapidly as soon as it was discontinued. This observation also undoubtedly reflects renewed proliferation of the surviving cells after treatment was stopped, as the less highly labeled ones began to fall below the counting threshold after resuming division.

The patient subsequently had a complete hematologic remission (text-fig. 5), and it is clear that most leukemic cells were killed by this course of treatment. Before therapy, the marrow was hypercellular and contained about 60% blasts, whereas after treatment it became very hypocellular and the proportion of blasts decreased to less than 3% (7/12/67). Hence it is certain that there was at least a 10-fold, and probably over a 100-fold, reduction in the number of leukemic cells (*43*). The exact fraction of cells killed could not be determined because we could neither accurately quantitate degrees of marrow cellularity nor distinguish between normal and leukemic blasts in the remission marrow. However, if one assumes that: *a*) There were roughly 10^{12} leukemic cells before treatment and 10^{10} after treatment; *b*) all surviving cells began to divide as soon as treatment was discontinued; *c*) their mean generation time was 4 days; and *d*) there was no cell death—then the population should have returned to roughly the original level in about 28 days, *i.e.*, after 7 doubles. Actually, on this occasion the remission lasted for about 2 months before marrow relapse was detectable (4% blasts on 9/11/67 to 35% on 10/2/67).

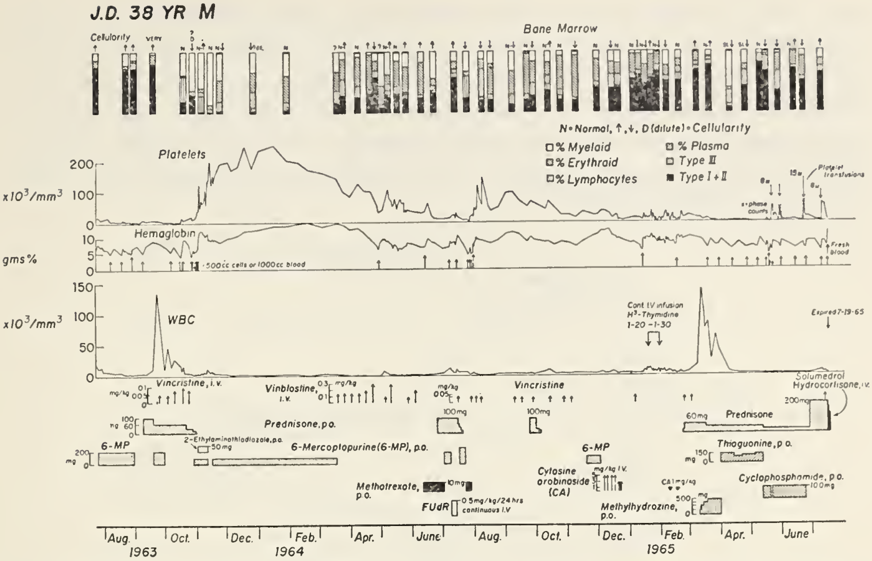
Therefore, evidently one or more of the above assumptions is not valid. Either: *a*) A greater than 100-fold reduction occurred; *b*) all surviving leukemic cells did not immediately begin to divide and continue dividing; *c*) their mean generation time was greater than 4 days; or *d*) a significant number died (or else matured to the extent of losing the ability to divide). We don't know which explanation is correct.

Although there are clear indications that such antimetabolites as arabinosyl cytosine can prolong the generation time immediately, we need more precise measurements of the effects of different cytotoxic drugs. The methods we have used for measuring changes during treatment are not very accurate, because the grain counts have often fallen too low by the time of measurement. However, if we start with too high a labeling intensity prior to therapy, the grain counts become too high to count reliably, and the proliferative kinetics may be altered by radiotoxicity. To obtain more accurate measurements of the effects of therapy by methods based on grain counts, repeated administration of ^{3}H-TDR will probably be necessary both before and during therapy.

Some drugs can also temporarily cause partial synchronization of cells. Short-acting antimetabolites in sublethal concentrations have been used extensively to produce such an effect in cultured cells. Shortly after stopping 5-fluoro-2′-deoxyuridine (FUDR) treatment of a patient with ovarian cancer (*44*), we observed a threefold to fourfold increase above the pretreatment value in the mitotic index of free-floating cancer cells in the ascitic fluid. The drug doses were sufficient to retard the passage of cells through the mitotic cycle and to prolong their generation time but were not lethal to most of the cells. Hence partial synchronization of the cells resulted after therapy was stopped. The effect was only temporary, however, as later in the patient's course the mitotic index and other proliferation parameters returned to their original pretreatment values. We also observed the ^{3}H-TDR labeling index of leukemic cells sometimes increases shortly after treatment with antimetabolites is discontinued (*2*). Partial synchronization is probably one explanation for this phenomenon. However, the effect *in vivo* is usually only partial and transient, and the cells quickly lose synchrony again. We have deliberately tried to synchronize acute leukemic cells by treating patients with low doses of methotrexate or FUDR, but these attempts were largely unsuccessful (*45*). Best results obtained were a twofold or threefold increase in the ^{3}H-TDR labeling index, and it usually returned to the pretreatment level within a week or two.

Delayed Effects of Chemotherapy

Treatment sometimes has a more lasting effect on the proliferation of leukemic cells. The duration of the effect undoubtedly varies according to the different mechanisms of action of the various drugs, but our data are far too incomplete to be able to make any such correlation. I mentioned earlier that in four of the five patients to whom we gave 8- or 10-day infusions of ^{3}H-TDR, about 90% of the leukemic cells were labeled, but that the fifth patient was an exception. This man (J.D.), with acute myelomonocytic leukemia, had been treated with numerous drugs for many months but had received no treatment in the 2 weeks preceding the ^{3}H-TDR infusion (text-fig. 11). His disease was in partial relapse but was progressing very slowly at the time of the study (*2*). His leukemic cells could be divided into three types based on their level of maturity (fig. 1). At the end of the 10-day infusion, most Type II and Type III cells were labeled in both marrow and blood, but only about 40% of the Type I cells



Text-figure 11.—Clinical course of patient J.D. The ^{3}H-TDR infusion study was done in January 1965.

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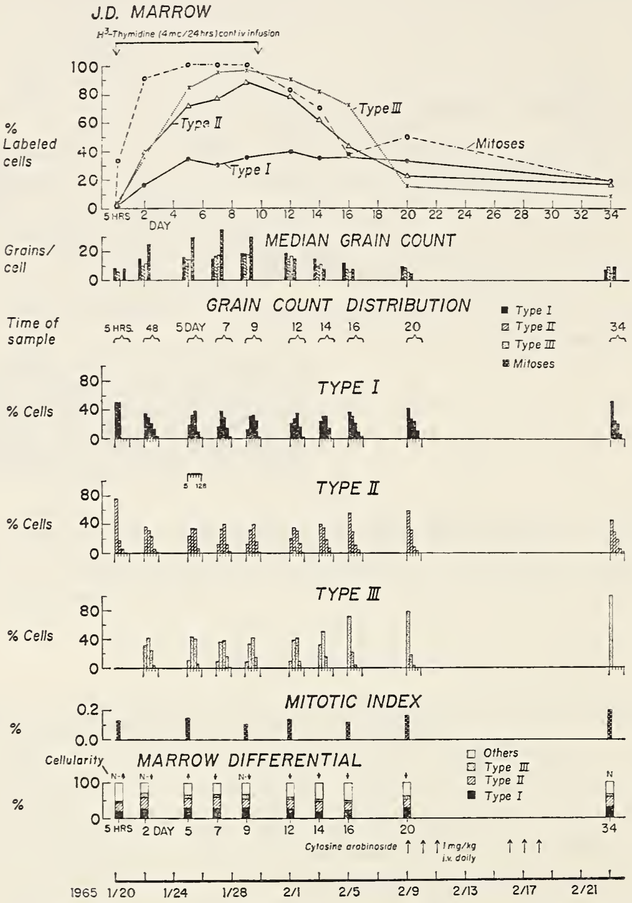
were labeled (text-figs. 12 and 13). Whereas the mean generation time of the Type II cells was 2 or 3 days, that of the labeled fraction of the Type I cells was about 8 days (*2*). Since about 60% of the Type I cells were dormant during the 10-day period, the mean generation time of the total Type I compartment was much longer than this.

The main reason for the slow growth rate of this patient's leukemia was therefore the very slow proliferative rate of the Type I cells. Even after some cells had left their dormant state and passed through one (or more) division cycles, some of their daughters must have again lapsed into a dormant state, since many of the Type I cells remained highly labeled for over 3 weeks after the infusion and the mean generation time of this labeled fraction was so long. However, once Type I cells started to mature and to become recognizable as Type II cells, they generally divided again fairly rapidly and their offspring did not persist for long. Some Type II cells matured further into Type III cells which no longer had the capacity to divide, while others passed into the blood as Type II cells where most of them presumably died. We could not determine the number of divisions occurring within the first two maturation compartments.

We cannot be certain that the slow growth rate of J.D.'s Type I cells was due to delayed effects from prior chemotherapy, but this seems probable, since in none of our other continuous infusion studies has such a large fraction of dormant cells been found. Although we did not do serial kinetic studies in J.D., both before treatment and later in his course, his leukemia showed rapid clinical progression (text-fig. 11). This suggests that even in him the delayed effect of therapy was only a relative matter, and that the surviving leukemic cells eventually recovered their original full growth potential.



TEXT-FIGURE 12.—Labeling pattern of leukemic cells in marrow of patient J.D. *See* text.

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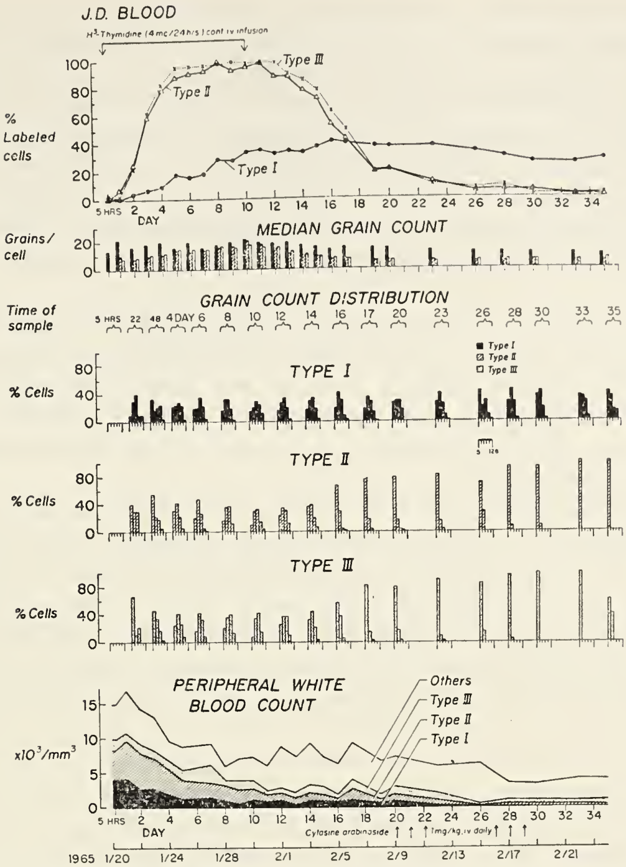

TEXT-FIGURE 13.—Labeling pattern of leukemic cells in blood of patient J.D. *See* text.

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INFLUENCE OF POPULATION DENSITY ON PROLIFERATION

High Population Density

Mammalian cells will grow *in vitro* only up to a certain maximum concentration, after which growth is inhibited (*46–55*). We have also observed this phenomenon with human lymphoblastoid cells in long-term suspension cultures (*38*, *56*). Of the latter cell lines, at least one is composed of neoplastic (reticulum sarcoma) cells, and one of non-neoplastic lymphocytes. The rest originated from the blood or spleen of patients with leukemia, but it is not known whether the cells in the established cultures are derived from leukemic cells or normal lymphocytes (*56–58*). In any case, whether their origin is normal, neoplastic, or uncertain, their proliferative behavior *in vitro* is similar. The cells do not die immediately after attaining their maximum concentration, and growth inhibition cannot be attributed to exhaustion of essential components in the medium since, if we then reduce the concentration of cells simply by removing some of them, the remaining cells will resume proliferation in the same medium (although not as well as in fresh medium). During exponential growth, their ^{3}H-TDR labeling index is about 50–60% and their mitotic index 1–2% or greater, while during the stationary phase these indexes decrease as does the amount of ^{3}H-TDR incorporated per cell (*59*). If the cells are not kept too long at a high concentration without feeding, their viability remains excellent and growth inhibition is reversible, since they will again begin to proliferate if they are diluted in either the same or fresh medium.

There is also evidence that the growth of experimental animal tumors slows progressively *in vivo* as the stage of the disease advances. Baserga and Gold (*60*) reported that the ^{3}H-TDR labeling index of Ehrlich ascites tumor cells decreased after the first few days of tumor growth and then promptly increased again when the cells from advanced tumors were transplanted into new hosts. Frindel, Malaise, Alpen, and Tubiana (*61*) found that the growth rate of an experimental murine fibrosarcoma slowed progressively with increase in cell number both *in vivo* and *in vitro*. In the latter sysem the duration of the cell cycle did not change during different stages of tumor growth. The slower growth rate resulted from a smaller growth fraction and increasing cell death. However, in Chinese hamster cells during their stationary growth period *in vitro*, other investigators found that the duration of both the G_2 and S phases was lengthened considerably (*55*), and we found the durations of S and G_1 to be greatly prolonged during the stationary growth period of our human lymphoblastoid cells (*59*).

Probably similar changes take place *in vivo* in the proliferation kinetics of human leukemic cells during different stages of the disease. When the marrow is densely crowded with leukemic cells, their rate of proliferation appears to be less than when many cells have been killed by therapy and their concentration has been reduced. There are many reports showing that the ^{3}H-TDR labeling index of acute leukemic cells frequently increases significantly above the pretreatment value when the disease relapses (*2, 3, 8, 24–26, 35*). Insufficient details regarding the temporal relation to treatment, type of drug used, and state of marrow cellularity were given in many of the reports to permit proper interpretation, and possibly the increased labeling index observed during relapse in some cases may have been due to factors other than a decrease in population density. Such factors would include: slowing of DNA synthesis in cells sublethally damaged by certain drugs, temporary partial synchronization of the cells in S after therapy was stopped, or a relative increase in the proportion of normal blasts which usually have a higher labeling index and which cannot ordinarily be distinguished from leukemic blasts (*3*). However, in some of the reports it seems fairly clear that a larger fraction of the leukemic population was proliferating during early relapse than when the marrow became packed with leukemic cells (*3*, *8*, *24*). Moreover, in several patients it was noted that the leukemic cells were significantly larger on the average during early relapse than when the disease was advanced, which is in keeping with their increased proliferative activity (*3*, *8*).

The reason for this "crowding" effect is not known, but we consider it is most probably due to elaboration by the cells of an inhibitory factor which reaches an effective concentration when they exist under densely populated conditions. Rubin and Rein (*46*) have presented evidence that inhibition of growth of cultured chick-embryo cells at high cell densities may be mediated either by a small dialyzable molecule or by local micromolecular changes in the environmental conditions. However, other investigators have evidence from different *in vitro* systems that density-dependent inhibition of cell division is not mediated by a freely diffusible substance but, rather, depends on direct intercellular contact (*47–54*). Thus, although cellular proliferation is unquestionably inhibited at high cell densities, the mechanism presently remains unknown, and it is also unclear how relevant the effects observed *in vitro* are to regulation of cellular proliferation *in vivo.*

Low Population Density

There is even less information regarding the growth rate of leukemic cells when very few of them are present. Although not yet proved, it seems probable that the small dormant leukemic cells usually survive treatment with most of the clinically useful drugs and eventually cause relapse of the disease. By means of serial *in vitro* determinations of mitotic and ^{3}H-TDR labeling indexes, we are now trying to determine the proliferative status of the residual blasts in patients who have recently been treated to the extent of severe marrow hypoplasia but in whom there are still a relatively high proportion of blasts which are almost certainly leukemic. We do not have enough data to draw any conclusions but hope eventually to answer the following questions:

1) Is it only the dormant cells that escape being killed by different drugs? 2) How soon do the cells start to proliferate after treatment with different drugs stops? 3) Is the growth fraction always greater and do they proliferate more rapidly after than before treatment?

If the dormant cells actually are the ones that survive, then clinical observations suggest that these surviving dormant cells (when not inhibited by the rest of the leukemic population) usually have the potential to grow at about the same rate as the original whole population. In most patients the leukemic cells appear to grow as rapidly when the disease relapses after remission as they did originally, and, as noted earlier, the leukemic population may even increase faster during early relapse before the cells become overcrowded. However, exceptions occur and the leukemic cells do not always proliferate at a constant and predictable rate.

In some patients with "preleukemia," relatively few abnormal cells may be present for months or even years (and may be associated with serious impairment of hematopoiesis) before enough accumulate to permit a diagnosis of leukemia (*62–64*). As to radiation-induced leukemia in atomic bomb survivors, an increased incidence of leukemia was not clearly evident for 18 months and did not reach a peak until 5 years after exposure (*65*, *66*). There is also the unexplained phenomenon that one occasionally sees of long unmaintained remissions in patients with acute leukemia after remission is induced with conventional chemotherapy (*e.g.*, G.S., the patient described earlier). There have been at least six patients at Memorial Hospital who presented with apparently typical acute myeloblastic leukemia whose remission, induced with conventional drugs, lasted 1 to over 3 years without maintenance chemotherapy before the disease relapsed. Burchenal reported similar instances of late relapses in patients with unmaintained remissions among his collected series of long-term survivors with acute leukemia (*67*).

If only one leukemic cell survived the initial course of treatment, after approximately 40 generations, 10^{12} cells would be present and relapse would certainly be recognized. If the mean doubling time was 4 days, relapse should therefore occur in 160 days, providing none of the cells died. Since none of our six patients initially received particularly aggressive treatment, it is almost certain that numerous leukemic cells were still present during remission. Hence the long remissions were most probably due either to a very slow rate of proliferation of the leukemic cells or to a high death rate (or maturation which would have the same effect). Unfortunately it has not been possible with available methods to study the proliferative behavior of leukemic cells during remission because they are rare and because usually they cannot be distinguished from normal blasts.

Reinduction of a new leukemic process cannot be excluded in these cases, but this is not likely. Several investigators have reported that in acute leukemia the leukemic cells are often aneuploid with chromosomal modes varying from patient to patient but apparently characteristic of each case (*68*, *69*). In remission the normal diploid mode is restored, whereas in relapse the original (individually characteristic) aneuploid stemline reappears.

Some cells will not multiply *in vitro* if they are present in too low a concentration, and the growth-enhancing activity of a "conditioning" factor, which is elaborated into the medium by actively proliferating cells, has been described (*46*, *70*, *71*). This factor will stimulate cells to divide when they are present in very low densities at which they could otherwise not initiate cell division. It has not been identified, but it appears to be a macromolecule, possibly a lipoprotein derived from the cell membrane (*46*, *70*). In our human lymphoblastoid cell lines in suspension cultures, single cells cannot initiate growth, although single cells from some lines can occasionally multiply in soft agar (*45*, *56*). When their starting density is only 100 (or in some lines 1,000) cells per ml of medium, some lines usually fail to grow, whereas others grow regularly at nearly exponential rates. Probably the various cell lines have different requirements for a growth-enhancing substance (or substances) before they will proliferate, but we have no direct evidence for this.

The marrow environment *in vivo* during leukemic remissions is of course much more complicated than that in culture. No information is available as to how the normal hematopoietic cells (or other unkown factors) might influence the growth of small numbers of residual leukemic cells, and this may well vary in different patients.

BEHAVIOR OF NORMAL HEMATOPOIETIC CELLS IN ACUTE LEUKEMIA

My final subject for discussion is the proliferative behavior of normal hematopoietic cells in acute leukemia. Our data are only fragmentary because most patients we studied had advanced disease and relatively few normal cells were present. We have conducted studies of varying degree of completeness of granulocytopoiesis and erythropoiesis in about twelve patients with acute leukemia and of lymphocytopoiesis in three patients. We have not yet studied megakaryocytopoiesis, mainly because there were usually not enough megakaryocytes for meaningful results. I will summarize our findings concerning erythropoiesis and granulocytopoiesis.

As to erythropoiesis, all the patients we studied were either already anemic or were becoming so when the studies were performed, and their total production of red cells was clearly deficient. Some patients had enough of the earliest recognizable red cell precursors (*i.e.*, pronormoblasts and early basophilic erythroblasts) to determine their labeling sequence, whereas others had only the more mature nucleated red cells present in sufficient numbers. The erythrocyte precursors were proliferating more rapidly in some patients than in others (as estimated by determining their initial and maximum ^{3}H-TDR labeling indexes, mitotic indexes, and the halving times of their median grain counts); but in almost all of the patients the cells appeared to be dividing and maturing at rates not very different from those reported for hematopoietically normal subjects by the Brookhaven group of investigators (*72–75*).

Precise comparisons are not feasible because the number of measurable parameters was limited. Moreover, too few normal subjects have been studied to determine the range of normal values. In some of our more complete studies (*4*), the median grain-count-halving times of the earliest red cell precursors were about a day or slightly less and these cells were dividing faster than the leukemic cells. However, there is evidence that in some patients the most rapidly dividing leukemic cells, *i.e.*, the largest ones, were probably dividing at about the same rate as the early red cell precursors. In all the continuous infusion studies, almost all nucleated red cells became labeled, providing their grain counts were high enough. We are presently examining the grain count data in more detail to determine the number of divisions occurring during intermediate stages of precursor development and the efficiency of erythropoiesis.

As to granulocytopoiesis in acute leukemia, we have measured the emergence times of labeled metamyelocytes, bands, and polymorphonuclears in the marrow and blood and found them to resemble those reported for normal subjects (*1*, *4*, *76*, *77*). Almost all granulocytes were labeled in all the continuous infusion studies by the end of the infusions (*4*). We usually could not determine the proliferative behavior of the earliest precursors, *i.e.*, normal myeloblasts and promyelocytes, because we could not distinguish them from leukemic cells or because too few were present, and we have found variable results regarding the initial labeling indexes of myelocytes and their median grain-count-halving times (about 2–4 days) (*1*, *4*). At present, because we lack data regarding the earliest normal granulocyte precursors in leukemia, and because of complicating factors such as infection and therapy in some patients, we cannot compare precisely the proliferative rate of granulocyte precursors or efficiency of granulocytopoiesis in leukemia with that reported in normal hematopoiesis (*76*–*79*). However, it does appear fairly certain that the remaining normal granulocytes in leukemia generally suffer no serious defect in maturation and that they mature at about the same rate as in normal subjects.

INTERRELATION OF LEUKEMIC AND NORMAL HEMATOPOIETIC CELLS

In untreated leukemia, the leukemic cells almost invariably compete successfully with the normal hematopoietic cells and eventually displace them. This seems to be mainly because the leukemic cells lose their ability to mature normally and thus many persist as primitive cells continuing to proliferate and accumulate in the marrow. The normal cells meanwhile continue to mature, and most of their progeny leave the marrow as functional cells. When enough leukemic cells accumulate in the marrow, they generally seriously disturb normal hematopoiesis. The total production of red cells and often of normal granulocytes may be greatly diminished, but the rates of proliferation and maturation of the remaining erythrocytic precursors and intermediate granulocytic precursors do not seem to be appreciably different from those in subjects with normal hematopoiesis. Since the proliferative rate of the normal precursors is almost certainly not slower than that of the leukemic population as a whole, the reduced production of normal cells must result from inhibition of the normal stem cells (*1*,*4*).

As I mentioned earlier, there is evidence that the leukemic population expands less rapidly when the cells are crowded together during advanced stages of the disease. Apparently the leukemic cells inhibit their own growth when their population density is great and also generally inhibit to an even greater extent normal stem cells. The degree in inhibition seems to vary considerably not only in different patients with the same type of leukemia but also according to the type of leukemia. For example, lymphosarcoma cells seem usually to inhibit normal hematopoiesis to a lesser extent than do acute myeloblastic leukemic cells. We have seen patients with a leukemic phase of lymphosarcoma whose marrows are diffusely and densely infiltrated with neoplastic cells but who are able to produce enough red cells, granulocytes, and platelets to maintain nearly normal blood counts, although their reserve capacity to respond to increased demand may be reduced. On the other hand, in myeloblastic leukemia, production of normal cells is often seriously impaired before extensive marrow replacement with leukemic cells occurs. Preleukemia may represent an extreme example of this phenomenon, but this syndrome is so variable and poorly understood that it is probably unwise to attempt any generalization about the reasons for impaired hematopoiesis in preleukemia.

The mechanism of inhibition is unknown, as is the reason that leukemic cells seem ordinarily less responsive to this form of growth regulation than are normal stem cells. Several possible forms of growth regulation have been described for various types of cultured cells (*46*, *47*, *49–53*, *80–83*), but none of these postulated mechanisms have been shown to have a physiologic role in controlling cell proliferation *in vivo.*

CONCLUSIONS

I hope I have helped clarify some aspects of the proliferative behavior of leukemic cells and their interrelation with normal cells, but many major unresolved problems remain. Since more attention should be focused on solutions, or at least on better definitions, of these problems, I shall discuss briefly the problems I consider most important.

The first problem concerns differences in the proliferation kinetics in different stages of the disease—especially after treatment, since most patients already have advanced disease at the time of diagnosis. For example, how regularly does the actively proliferating fraction of surviving cells increase after an initial course of treatment which kills most of the leukemic population? Is the mean generation time of the surviving cells shorter than that of the original whole population? Is the generation time of these residual cells less variable? Do they divide more synchronously and, if so, how long does synchrony persist? Is their rate of DNA synthesis faster than when they are densely crowded? How soon do the surviving cells begin to proliferate after treatment with different drugs is discontinued? And are these changes in proliferation kinetics sufficiently consistent and predictable in different patients after treatment with particular drugs to enable us to take advantage of them to formulate more effective treatment schedules?

The second problem concerns a clearer understanding of the dormant state. Not only is this of fundamental biological interest, but if the cells that usually survive intensive chemotherapy are indeed the dormant cells, it would obviously be of primary importance to develop ways to destroy these cells before they can begin to proliferate and repopulate the marrow. Schabel, Skipper, and co-workers have extensive data showing the relative concentrations of different drugs needed to sterilize both dividing and resting L1210 mouse leukemic cells (*84*).

If we can define more precisely the behavior of dormant human leukemic cells, it may be possible to apply more effectively to the human disease some of the principles that have been so carefully worked out in the mouse model, although I strongly suspect that the temporarily resting L1210 cell is quite different from the long-term dormant human leukemic cell. From presently available information (*84, 85*), one would anticipate that the best time to administer a large dose of a drug highly lethal to resting cells (*e.g.*, 1,3-bis(2-chlorethyl)-1-nitrosourea or BCNU) would be immediately after a course of treatment with other drugs that had killed most of the actively dividing cells. However, it is generally just at this stage that the marrow is most depressed and the patient is least able to tolerate further treatment. Possibly, if one can induce remission with an agent such as L-asparaginase (*86*), which does not appreciably damage the normal hematopoietic cells, one can then give large enough doses of another drug (or drugs) to kill all residual dormant cells (or else try to reverse the sequence).

It is also most important to learn how the dormant cells differ biochemically from the actively proliferating cells. If this were better understood, methods might be devised to force the dormant cells to reenter the division cycle at an appropriate time, so that they can then be killed by drugs active against proliferating cells.

The final problem involves the central enigma of the nature of the essential defect (or defects) in leukemia, *i.e.*, not only is it unknown why leukemic cells have lost their ability to mature normally but there is the associated (if not related) problem of why they are usually less responsive to growth inhibition than are normal hematopoietic stem cells. Leukemic cells may, to a variable degree, simulate the behavior of normal stem cells and their progeny. Like normal cells, some leukemic cells die, some may mature, and others stay dormant for extended periods. Moreover, their rate of division is generally of the same order of magnitude, and their response to an increased death rate appears to be similar in that the fraction of actively dividing cells may increase. However, it is obvious that leukemic cells do not conform to a normal, orderly growth pattern. They have, at best, a limited ability to mature, they can continue to proliferate (at least slowly) at population densities so great that normal stem cells are almost completely inhibited, and their birth rate is almost inevitably eventually greater than their death rate, so that a steady state is not maintained. In our efforts to comprehend the more easily soluble problems, we must not disregard continuing research to discover the nature of the fundamental flaw which must underlie their anomalous behavior.

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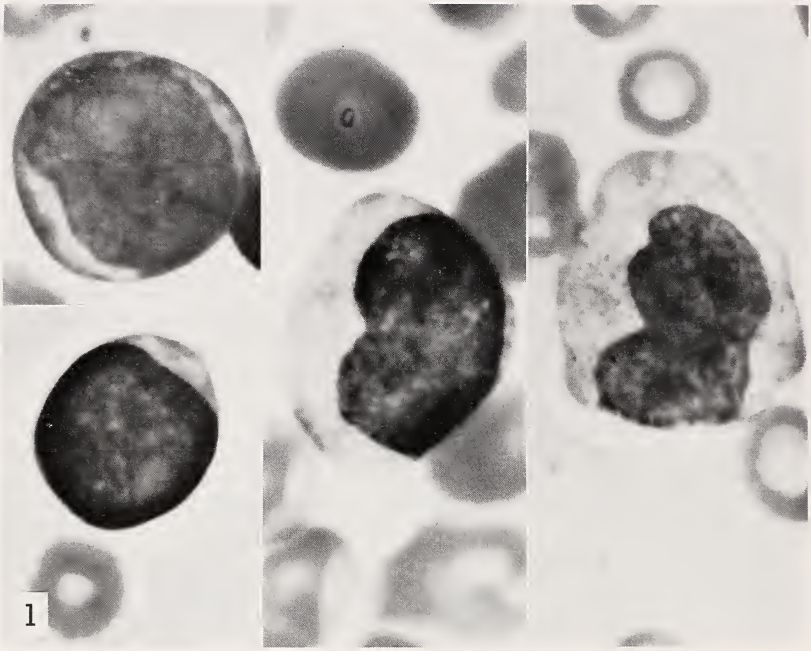


Figure 1.—Leukemic cells of differing levels of maturity from patient J.D. with acute myelomonocytic leukemia. From *left* to *right:* Type I = most primitive blast form recognized (two different sized cells are shown) ; Type II = intermediate form; and Type III = most mature form. × 12,000

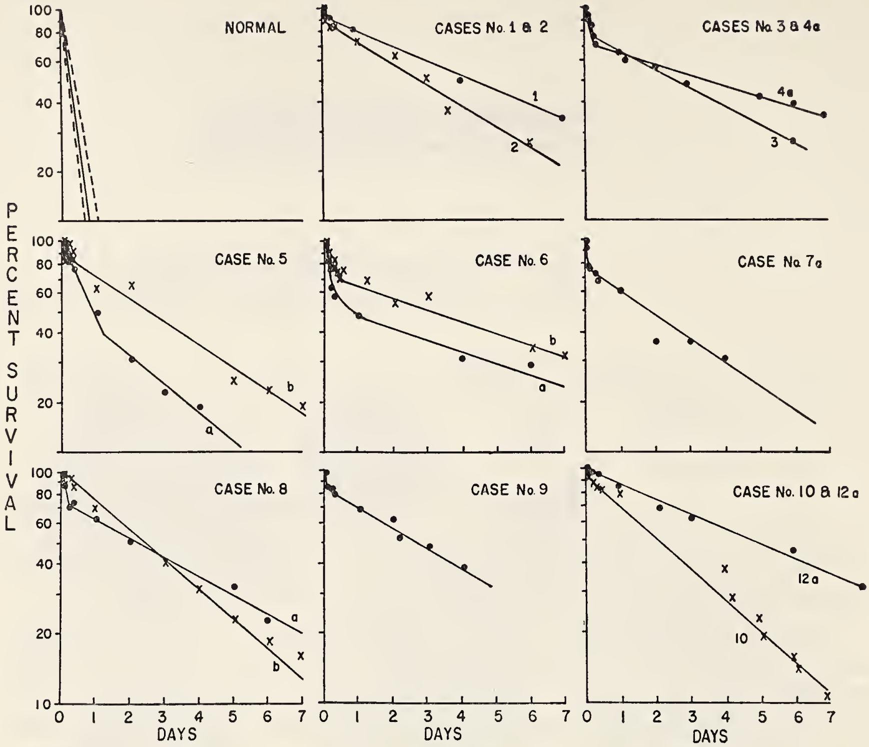
Granulocyte Kinetic Studies in Chronic Myelogenous Leukemia

Peter R. Galbraith, *M.D.*, *FRCP(C)*,
Queen's University, *Kingston*, *Ontario*, *Canada*

THIS PAPER summarizes the results of some of our recent investigations and attempts to separate fact from fancy in a discussion of their possible relevance in the understanding of one neoplastic disease, chronic myelogenous leukemia (CML).

Granulopoiesis in CML has been investigated by a leukocyte kinetic approach in which leukocytes were labeled *in vitro* with radioactive diisopropylfluorophosphate ($DF^{32}P$) and returned to the circulation (*1*, *2*). Leukemic leukocytes thus labeled disappeared at an abnormally slow rate, but their mode of disappearance varied greatly and depended to a large extent on the hematological status of the patient (*1*,*2*) (text-figs. 1 and 2). When the subject was in relapse, most of the leukocytes left the circulation at a slow exponential rate and, when the patient was in remission, most of the cells disappeared at an exponential rate comparable to that found in studies of normal subjects. Two things obscured the significance of these findings: 1) The blood leukocyte population in leukemic subjects was complex with respect to their level of maturation, and 2) most of the leukocyte disappearance curves were complex; *i.e.*, in relapse, a small proportion of the leukocyte radiactivity disappeared from the blood at a fast rate and, in remission, a small proportion of the leukocyte radioactivity disappeared slowly.

In our initial studies, a leukocyte fractionation procedure was used to obtain mature leukocytes (PMN) from the complex population of leukocytes harvested during leukocyte kinetic studies (*2*) (text-fig. 3). Hence, it was possible to examine the levels of specific activity in circulating mature leukemic neutrophils (text-fig. 4). It was ascertained that the lifespan of leukemic PMN was greatly prolonged (*2*). This finding explained the observation that a single, slow exponential curve could describe the disappearance of a complex leukocyte population. Presumably the labeled myelocytes can undergo maturation, and thus enter the "functional" pool without appreciably affecting the slope of the curve.



Text-figure 1.—Leukocyte specific activity curves in subjects with chronic myelogenous leukemia in relapse.

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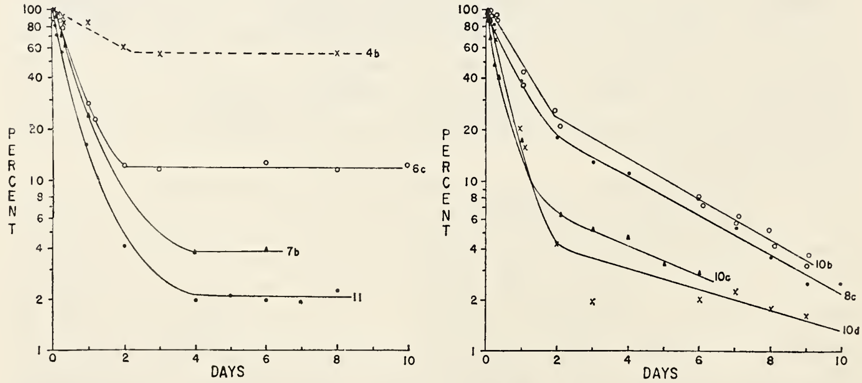

Text-figure 2.—Leukocyte specific activity curves in seven subjects with chronic myelogenous leukemia in remission, partial remission, or partial relapse.

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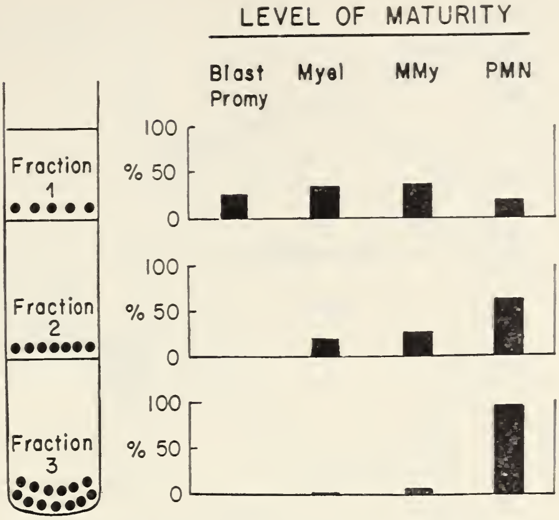


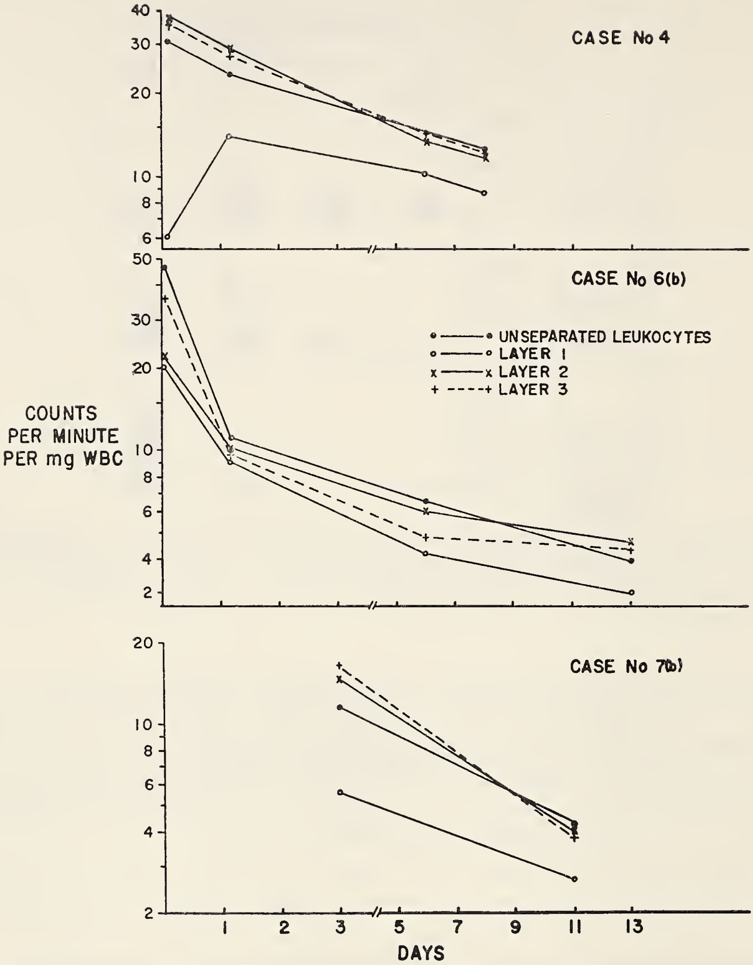
TEXT-FIGURE 3.—Mean composition of leukocyte fractions obtained by density gradient centrifugation of blood leukocytes harvested during leukokinetic studies. Corresponding levels of leukocyte specific activity in the fractions are shown in text-figure 4.

An attempt was then made to determine if the long lifespan of the leukemic neutrophils was due to an intrinsic abnormality or to extracorpuscular factors. This was approached in the classical way, by a series of crosstransfusion experiments. Homologous normal leukocytes obtained from professional donors were transfused into patients with CML in relapse. The disappearance of the normal leukocytes was not retarded by extracorpuscular factors (*2*) (table 1). In the reverse experiment, in which leukocytes from patients with CML in relapse were transfused into recipients with nonleukemic malignant disease, the labeled cells disappeared from the blood at a changing rate. The initial disappearance of the leukemic cells occurred at a rate faster than normal, but subsequent loss occurred more slowly (text-fig. 5).

These findings suggested that the neutrophils in CML have an intracorpuscular defect which permits them to have a long intravascular lifespan, but that extracorpuscular factors influence to a large extent the realization of this potential. That the abnormal cells were sequestered

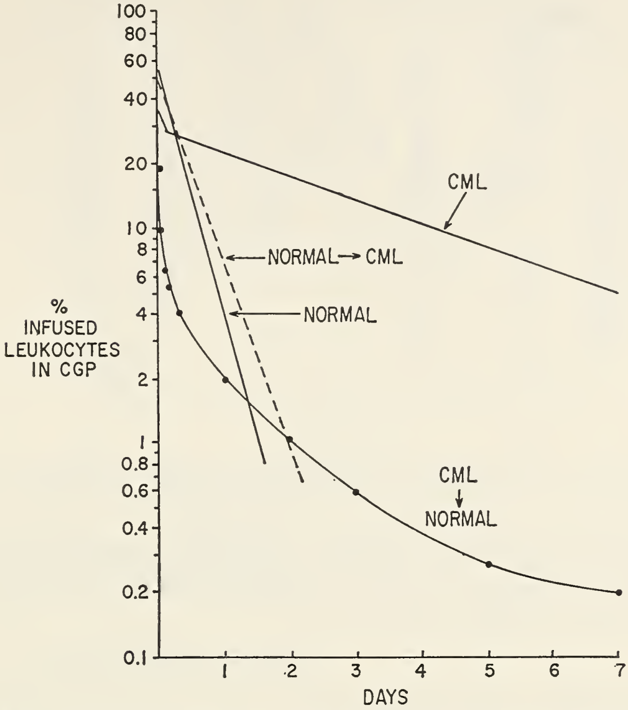
TABLE 1.—Infusion of homologous normal leukocytes into subjects with chronic myelogenous leukemia in relapse

Number of studies, 5	$T\frac{1}{2}$(hr)	
	Autologous	Homologous
Mean	80	7.78
Range	(62–88)	(5.4–9.2)



Text-figure 4.—Leukocyte fractionation studies. Specific activity of leukocytes in fraction 1 (○——○) composed mainly of immature cells, fraction 2 (X——X) composed of a mixture of immature and mature cells, and fraction 3 (+——+) composed of mature neutrophils is compared with the specific activity of unseparated blood leukocytes (●——●) during leukokinetic studies. Mean composition of the leukocyte fractions is shown in text-figure 3.

from the circulation quickly suggested itself, as did other questions pertaining to the return of the leukocyte disappearance curves to normal in CML in remission. For example, compartmental analysis of the complex curves suggested that the fast component, like the slow component, resembled a single exponential function (text-fig. 6). Thus, if only one kinetic population of leukocytes was present in the blood of the leukemic patients, the fast component might simply represent a mixing phenomenon in which the equilibration of the labeled cells between an intravascular and

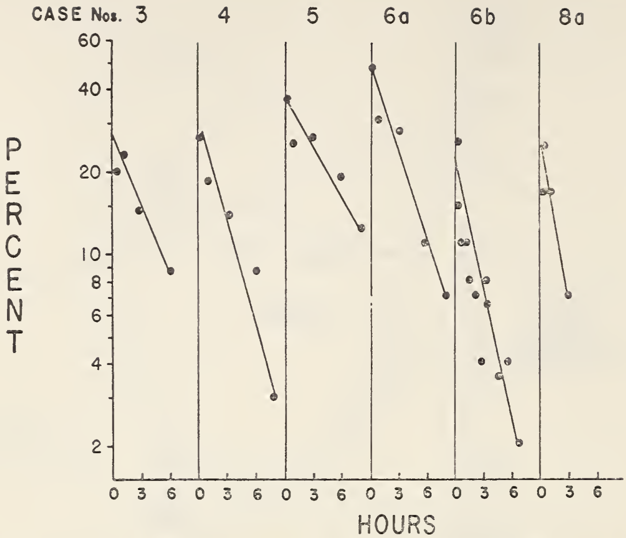


TEXT-FIGURE 5.—Composite diagram showing manner of disappearance of leukemic and nonleukemic leukocytes in autologous studies and in crosstransfusion studies. The curve of disappearance of the leukemic leukocytes from the circulation of nonleukemic recipients was obtained by pooling data from eight studies. CML=chronic myelogenous leukemia.

extravascular compartment was delayed, and the actual lifespan of the leukocytes could be described by the slow component.

Problems related to possible sequestration of leukemic leukocytes seemed of such fundamental importance to our understanding of the leukocyte disappearance curves that we proceeded to study the exchange of *in vitro*-labeled leukocytes between the blood and one accessible extravascular compartment, the bone marrow. A simple method was devised, which permitted the isolation of myeloid leukocytes from bone marrow particles (*3*) (text-fig. 7). The leukocytes thus isolated were free from contamination by labeled leukocytes in peripheral or sinusoidal blood. Thus, in studies in control subjects, *in vitro*-labeled leukocytes, infused into the circulation, could not be detected in the bone marrow parenchyma, though they were present in leukocytes isolated from simple marrow aspirates (text-fig. 8) (*3*).

In two patients with CML in relapse, after infusion of labeled cells, the label quickly equilibrated between the blood and marrow leukocyte compartments (text-fig. 9). Complete equilibration of the label suggests that leukocytes at all levels of maturation participate in the exchange.



TEXT-FIGURE 6.—Isolated fast component of leukocyte survival curves in patients with chronic myelogenous leukemia in relapse obtained by compartmental analysis of the complex curves.

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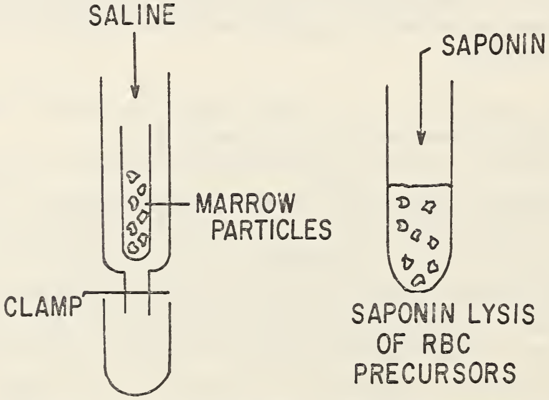
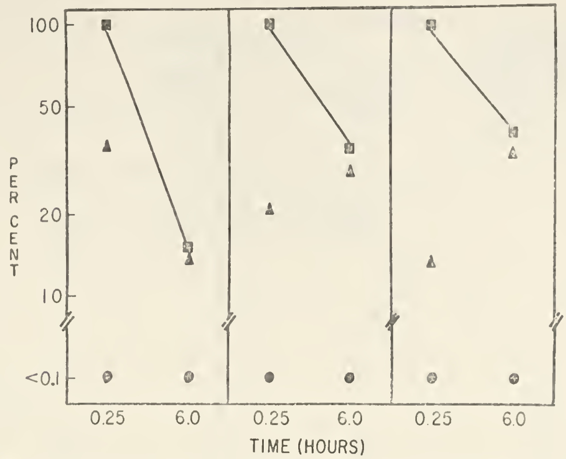


TEXT-FIGURE 7.—Method for obtaining leukocytes from bone marrow parenchyma. Aspirated marrow was passed through a standard blood administration set and washed. Marrow particles were broken up inside the filter and leukocytes from them collected separately. Erythroid precursors and erythrocytes were lysed with saponin, and the myeloid cells thus isolated were free from contamination by circulating leukocytes.



Text-figure 8.—Levels of leukocyte specific activity in leukocytes isolated from the blood (■——■), bone marrow aspirates (▲), and bone marrow parenchyma (●) after the infusion of autologous normal leukocytes labeled *in vitro* with DF^{32}P. Results are expressed as a percentage of the blood leukocyte specific activity 15 minutes after completion of the infusion.

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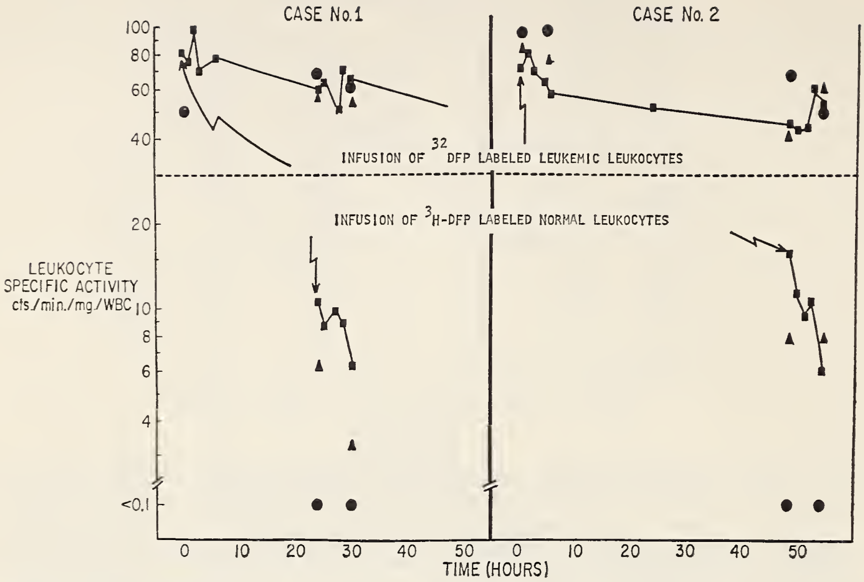
The use of this technique in the studies on the leukemic subjects was validated by the demonstration that homologous normal leukocytes labeled *in vitro* with ^{3}H-DFP did not enter the bone marrow parenchyma, though they were recovered from marrow aspirates (text-fig. 9). In two patients with CML in partial remission and in one patient in complete remission, *in vitro*-labeled autologous leukocytes could not be detected in the bone marrow parenchyma (text-fig. 10) (*4*). This series of findings suggests that exchange of the leukocytes between the blood and bone marrow is determined by intracorpuscular factors rather than by anatomical changes in the bone marrow, and that the intracorpuscular factors leading to the exchange are absent in remission.

Further evidence was sought to determine if the kinetic properties of the leukocytes are different in subjects in relapse and remission. We have done crosstransfusion studies in which one patient with carcinoma served as the recipient of leukemic leukocytes obtained from the same leukemic donor in relapse and later in remission (text-fig. 11). In remission, the leukemic leukocytes disappeared from the blood of the nonleukemic recipient in a manner indistinguishable from the normal, and there was no evidence of sequestration (*5*). This finding has recently been confirmed in two further studies.

Thus, "nonsequestration" and "nonexchange" may reflect an improvement in leukocyte function in CML in remission. This suggestion is supported by the recent findings of Penny and Galton (*6*) that *in vitro* phagocytic activity of leukemic PMN improves in remission.



Text-figure 9.—Studies on the exchange of leukocytes between blood and bone marrow in 2 patients with chronic myelogenous leukemia in relapse. Levels of specific activity in leukocytes isolated from the blood (■——■), bone marrow aspirates (▲), and bone marrow parenchyma (●) after the infusion of autologous leukemic leukocytes labeled *in vitro* with $DF^{32}P$ (*above dashed line*) and after the infusion of homologous normal leukocytes labeled *in vitro* with ^{3}H-DFP (*below dashed line*). (The leukemic leukocytes returned to the bone marrow parenchyma, but normal leukocytes could not be detected in bone marrow parenchyma.)

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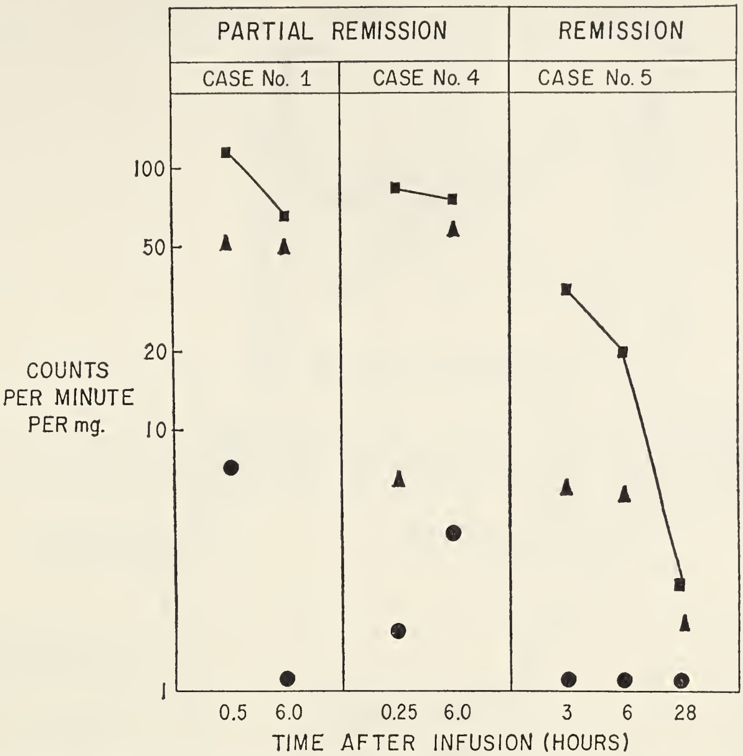
Although the circulating PMN in CML in remission arise from a stem cell line bearing the Philadelphia chromosome (*7*), "nonsequestration" and "nonexchange" of such leukocytes set them apart from the cells encountered in relapse, and suggest that the concept that the blood contains a single kinetic population of PMN needs to be reevaluated. The evidence suggests that the biphasic leukocyte disappearance curves encountered in partial and complete remission are now best explained on a two (or more) population basis.

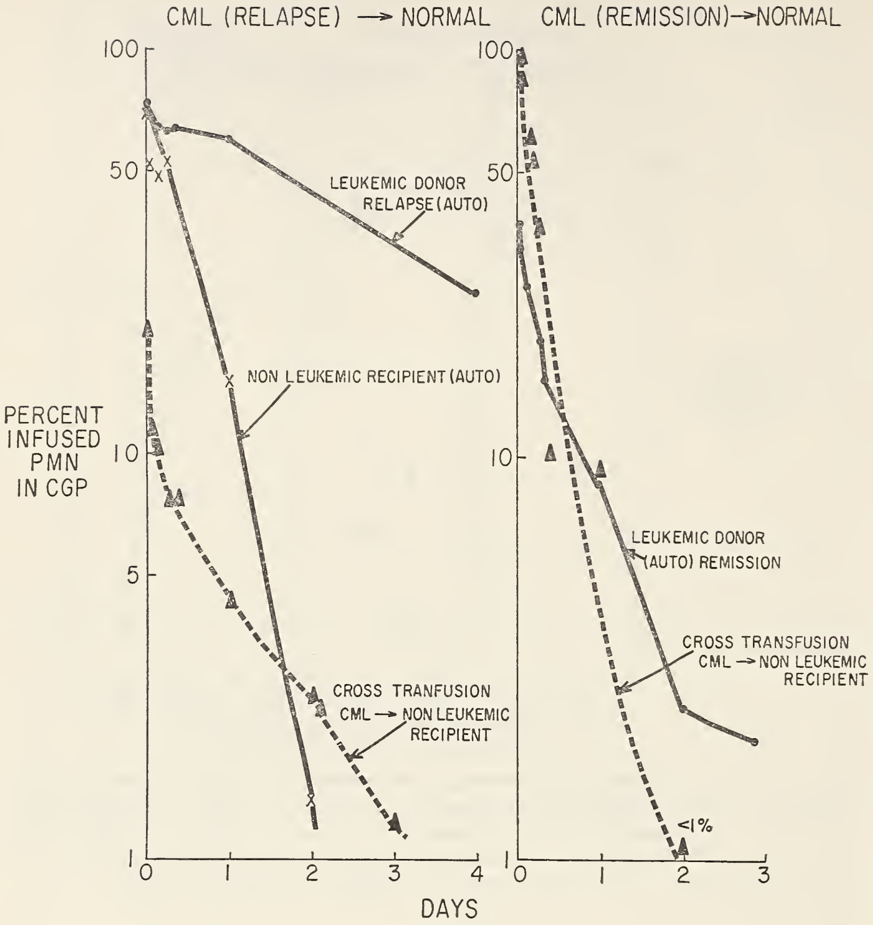
Even though autologous studies in patients in remission indicate that the leukocytes disappear from the blood at a normal rate, the potential for prolonged survival of these cells in the presence of appropriate extracorpuscular factors is illustrated in a crosstransfuson study, in which the labeled leukocytes of a patient with CML in partial remission were transferred into a patient in relapse (text-fig. 12) (*2*). By this maneuver, it was found that a large percentage of the donor leukocytes had the capacity for prolonged survival. It was also observed that the disappearance of the leukocytes obtained from the subject in relapse was accelerated by transferring them to the patient in partial remission (*2*).



TEXT-FIGURE 10.—Studies on the exchange of leukocytes between blood and bone marrow in 2 patients with chronic myelogenous leukemia in partial remission and 1 patient in full remission. Levels of specific activity in leukocytes isolated from blood (■——■), bone marrow aspirates (▲), and bone marrow parenchyma (●) after the infusion of autologous leukocytes labeled *in vitro* with DF^{32}P.

In summary to this point, the data suggest that the leukocytes in CML are defective. They have a long intravascular lifespan and tend to be sequestered into extravascular compartments, from which they recirculate. In remission, leukocyte function improves, in the sense that the cells can avoid sequestration, but the potential for a prolonged lifespan is retained. The possible signficance of these findings will now be discussed.

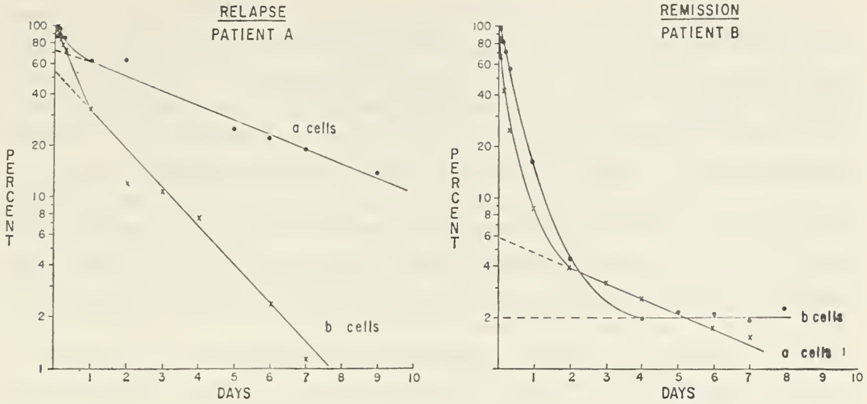
Clearly one of the important abnormalities in granulopoiesis in CML is the potential of the leukemic cells for an extraordinarily long life. It follows that the massive granulocytosis, which is characteristic of the disease, may be achieved at a lower level of granulocyte production than would be expected in the absence of this lesion. The granulocyte turnover data reported by Athens and colleagues (*1*) are in accord with this concept. These workers reported one case in which the granulocyte turnover rate (GTR) was only 6 times the upper limit of normal, and the remainder of their cases had GTR's equal to or less than 3 times the upper limit of normal. We have made similar calculations from our own data



Text-figure 11.—Crosstransfusion studies performed between one leukemic donor and one nonleukemic recipient. When the donor was in relapse, very few leukocytes appeared in the circulating granulocyte pool and these were rapidly removed from it. When the leukemic donor was in remission, his leukocytes were not sequestered by the nonleukemic recipient. CML = chronic myelogenous leukemia.

and have found patients in whom granulocytosis was present in the absence of an elevated GTR (text-fig. 13).

For the prolongation of the lifespan to be manifest, appropriate extracorpuscular conditions must be present. The leukemic PMN apparently must find themselves in a greatly expanded total blood granulocyte pool (TBGP). How, then, can modest increments in the rate of leukocyte production result in expansion of the TBGP? The biphasic leukocyte disappearance curves observed in patients in remission probably give an important clue with which to answer this question—the clue that there may be two or more populations of leukocytes present in the blood, *e.g.*, populations A and B. Population A, denoted by the fast component of the



Text-figure 12.—Leukocyte specific activity curves obtained when leukocytes of a patient with chronic myelogenous leukemia in partial remission were transfused into a patient with chronic myelogenous leukemia in relapse, and vice versa. Curves obtained following the infusion of labeled autologous cells are shown for comparison.

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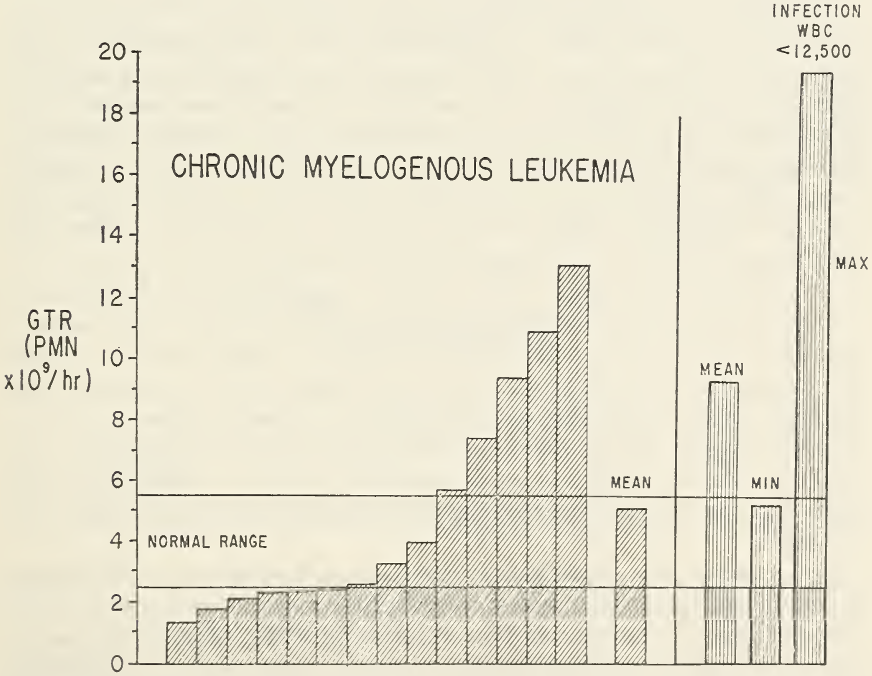

Text-figure 13.—Granulocyte turnover rate (GTR) in chronic myelogenous leukemia in relapse. The values given refer to mature (bands and segmented) forms only. Limitations in the accurate estimation of the GTR are discussed in the text. With these reservations, it appears that granulocytosis can occur in the absence of an increased rate of granulocyte production in leukemic subjects.

curve, consists of nonsequestering, nonexchanging PMN which disappear from the blood at a normal rate, and whose kinetic abnormality is only manifest when the TBGP is very large. This population by itself is probably not capable of inducing granulocytosis when there are only modest increases in the rate of granulocyte production. Population B, denoted by the slow component of the curve, appears to consist of a population of cells whose long lifespan does not depend on the expansion of TBGP. Population B probably consists of cells, which, like those encountered in patients in relapse, are sequestered to a large extent in extravascular sites. Sequestration of such cells may thus give them an added biological advantage as they are removed from the risk of random utilization in the circulation. One can hypothesize that it is the emergence of population B which is responsible for the primary expansion of the TBGP and the development of recognizable disease.

It is difficult to understand the variations in the pattern of disappearance of leukemic leukocytes when they are transferred from one environment to another, as in crosstransfusion studies, in the absence of a satisfactory concept concerning the factors governing granulocyte utilization. The problem becomes more complex because we are dealing with complex populations of leukocytes having a spectrum of abnormalities.

For purposes of discussion one can suppose that the rate-limiting factor governing the total number of PMN which leave the circulation per unit time is the number of exit sites through which migration is possible. It is known that the migration of a neutrophil from a blood vessel into an inflammatory focus requires its margination and its active interaction with the vascular endothelium through which it migrates. Since leukemic neutrophils migrate poorly into exudates (*8*) and are defective in other ways, it is likely that when two populations of neutrophils coexist, those with normal function will compete more favorably for the available exit sites. Possibly the presence of one kinetic population may influence the rate of disappearance of the other under conditions where the number of exit sites is rate limiting. It is also possible that, under conditions of exit site excess, the presence of multiple populations may be, to some extent, masked, since the element of competition is removed. The observation that homologous normal PMN, transfused into the blood of patients with CML in relapse, disappear at a normal rate can be explained on the basis that they compete more successfully than the leukemic PMN at the exit sites.

The presence of multiple kinetic populations of leukocytes in the blood of leukemic patients again raises speculations concerning the role of the Philadelphia chromosome in the evolution of CML. That this is an acquired cytogenetic abnormality seems beyond doubt, since it is found only in hematopoietic cells (*9*) and has been reported in one member of a pair of monozygotic twins on at least three occasions (*10–12*).

Also, Weiner has studied a family with high incidence leukemia. The Philadelphia chromosome was found in blood metaphases of family members who did not have clinical leukemia (*13*). Possibly the emergence of a stem cell line bearing the Philadelphia chromosome constitutes a "minimum deviation," to use Potter's term (*14*). This clone of cells may expand to fill the bone marrow because it is not sensitive to negative feedback mechanisms and may, in fact, be able to exert feedback inhibition of normal granulopoietic elements. Possibly, clinical leukemia does not become manifest until, through random mutation, further deviation occurs with the emergence of more abnormal stem cell lines whose progeny show diminished functional capacity, and thus they accumulate. Ultimately, further escalation of the malignant process culminates in the myeloblastic transformation.

There are two further implications suggested by the data which I have presented. First, it may not be possible to make accurate estimates of the size of the TBGP and the rate of granulocyte turnover in CML because of the presence of multiple populations of neutrophils, some of which are not confined to the intravascular compartment. Thus, in CML in relapse, one may at best measure the total exchangeable granulocyte pool rather than the number of cells confined to the intravascular compartment. Second, the difference in the distribution of the two populations may be the reason that focal splenic irradiation is so effective in the treatment of this disorder. The population B, which exchanges between the blood and bone marrow, probably also exchanges with cells in the spleen. Irradiation of this reservoir may thus preferentially eliminate population B cells from the blood and bone marrow (*15*). Finally, I would like to emphasize that concepts of granulopoiesis in CML must come under constant review as new data become available. The views I have expressed are those which I hold at the moment and those which I have found useful in interpreting the data.

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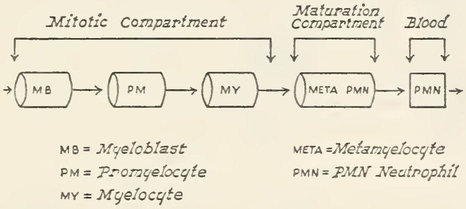
Granulocyte Kinetics in Health and Disease

John W. Athens, *Hematology Division, Department of Internal Medicine, The University of Utah, Salt Lake City, Utah 84112*

First I will review our studies of normal granulocyte physiology and kinetics and then describe some of the things we have tried to do in certain disease states.

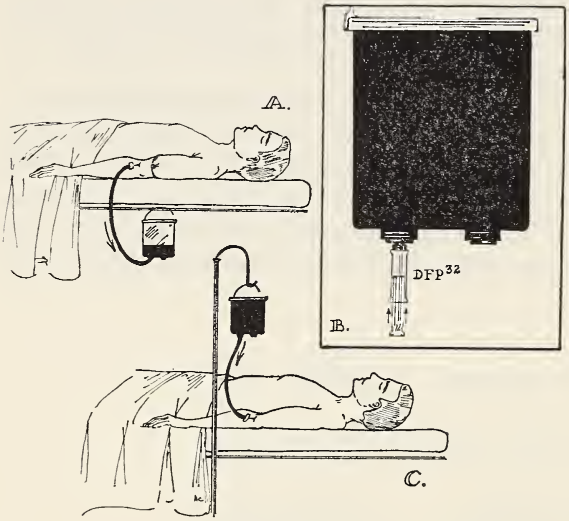
A crude model of most of the life of a granulocytic leukocyte is shown in text-figure 1. The life of the granulocyte can be divided into three phases: 1) the marrow phase, 2) the blood phase, and 3) the tissue phase. Only the marrow and blood phases are shown in text-figure 1; the tissue phase is off the text-figure to the right, omitted because we know little about it. For that reason, we really don't know what the lifespan of the granulocyte is.



Text-figure 1.—Model of granulocyte lifespan.

The marrow phase of the granulocyte lifespan is divided into two general areas: 1) the mitotic compartment containing the precursor cells capable of cell division, *i.e.*, myeloblasts, promyelocytes, and myelocytes, and 2) the marrow granulocyte reserve or maturation compartment, which is composed of the cells unable to divide and undergoing a maturing process. This compartment includes metamyelocytes, bands or juveniles, and segmented mature neutrophils. Cells move through these stages sequentially and into the blood.

In describing our studies, I will begin with the blood compartment and work back into the marrow compartment. Text-figure 2 shows one of the two techniques we used to study granulocyte physiology. We call this technique the *in vitro* technique because blood from the individual is put in a plastic transfusion bag and labeled *in vitro* with radioactive diisopropylflourophosphate (DFP). DFP is added to the blood in the bag, labeling being complete in about 45 minutes. Then the labeled blood can be injected back into the donor. During the hour of incubation, the DFP binds to the blood cells, predominantly to neutrophilic granulocytes. After an hour, a measured amount of labeled blood is given back to the individual, and various studies are carried out. Early in our work, we demonstrated that, once the label was bound to the cells, there was no label elution and the labeled cells were not damaged in any way. In addition, we developed a method for collecting and separating leukocytes, actually almost pure granulocytes; this method enabled us to follow granulocyte radioactivity.

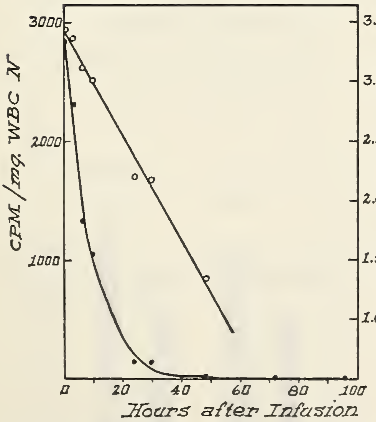


Text-figure 2.—*In vitro* method.

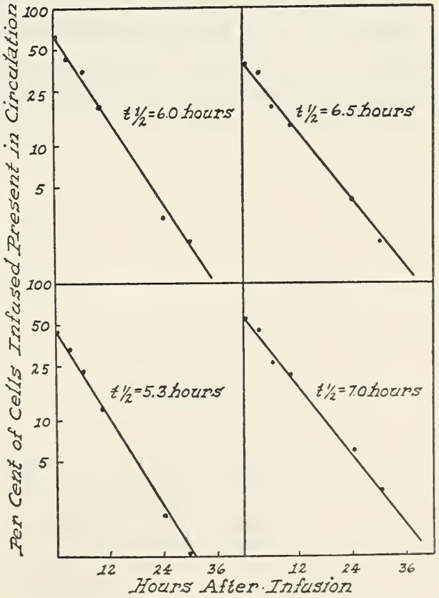
Figure 1 shows, by means of autoradiography, with the use of tritiated DFP, that neutrophilic granulocytes label heavily with DFP (*1*). The grains produced by the ^{3}H-DFP are over the cytoplasm of the segmented and band forms, and there is no labeling of red cells, eosinophils, or lymphocytes, and minimal labeling of monocytes. An autoradiograph obtained from DFP-labeled chronic myelocytic leukemia blood is shown in figure 2. The labeling of segmented and band forms is evident, and metamyelocytes and myelocytes are also labeled, but blast forms are not. There is some debate in our group as to whether promyelocytes label. In dogs, promyelocytes probably do not label, and my colleague, Dr. Boggs, feels certain of this. In humans, some later stages of the well-granulated promyelocytes may label to the same degree as myelocytes.

If one takes labeled blood from an individual, labels it by the method described, returns it to his circulation, and then obtains samples of blood at intervals thereafter, separates the leukocytes, and measures the blood-granulocyte radioactivity, one gets the curve (solid circles) shown in text-figure 3, which seems to be an exponential curve. If one plots the same data on a logarithmic scale (hollow circles), a straight line is obtained. When we first obtained these results, we immediately searched again for elution and cell damage. No evidence of either was found, and we concluded that neutrophilic granulocytes leave the blood in a random, or exponential, manner rather than according to their age, as do erythrocytes. This may be one of the points Dr. Perry was referring to when he said the work has managed to stand up over the years because we, he, and many others really had a hard time believing this. However, evidence from other studies, including the time course of cells labeled *in vivo* in the circulation, either with DFP or tritiated thymidine, corroborates this finding.

In addition to being able to follow the disappearance curve of labeled cells from the blood, we thought we should be able to measure the blood-granulocyte pool size, just as one measures the red blood cell mass with radioactive chromium. When we tried to do this and plotted the percent of cells infused which could be recovered in the circulation at the end of the infusion (text-fig. 4), we were disturbed to find that only about 50% of the cells infused could be accounted for in the blood.



TEXT-FIGURE 3.—Arithmetic and semilogarithmic curves of DFP-labeled blood-granulocyte disappearance.



TEXT-FIGURE 4.—Four semilogarithmic *in vitro* blood-granulocyte radioactivity curves (2).

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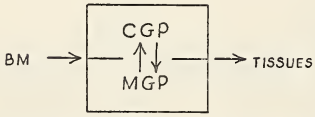
This again raised the questions of cell damage and label elution, and again, we could not demonstrate either. Therefore, we were led to suggest that perhaps the pool in which the injected cells were distributed was larger than the product of the blood volume and the granulocyte count. The simple model shown in text-figure 5 illustrates this. We hypothesized that the total blood granulocyte pool, represented by the box, consisted of two compartments, a circulating granulocyte pool (the blood volume × the granulocyte count), and an undefined and not well-localized marginal granulocyte pool, cells perhaps sticking along the walls of blood vessels in various organs throughout the body. This idea is not original with us. As early as 1860 eminent workers in various biologic sciences had observed cells sticking along the walls of small blood vessels, reentering the circulation, and being replaced by cells from the circulation which then stuck to the walls of the vessels.

To test this hypothesis we carried out further studies, one of which is illustrated in text-figure 6. We labeled blood from an individual in the manner just described, returned it to his circulation, and determined the percent of infused cells present after completion of the infusion. In a group of 5 individuals, we found it was about 50% as is illustrated in the bars labeled "before" in text-figure 6. Then we gave these same individuals a rapid infusion of epinephrine, which resulted in a rise in blood-granulocyte concentration to about twice the control level. At the peak of that granulocytosis, we could account for an average of 72% of the cells infused (text-fig. 6). In similar experiments we were able to mobilize even more of the cells by exercising the experimental subjects. A quarter-mile run increased their white counts rather markedly and increased the number



Text-figure 5.—Model of the blood-granulocyte pool. Figure 4 in (*3*).

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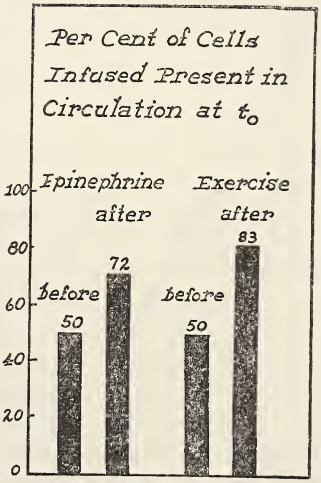
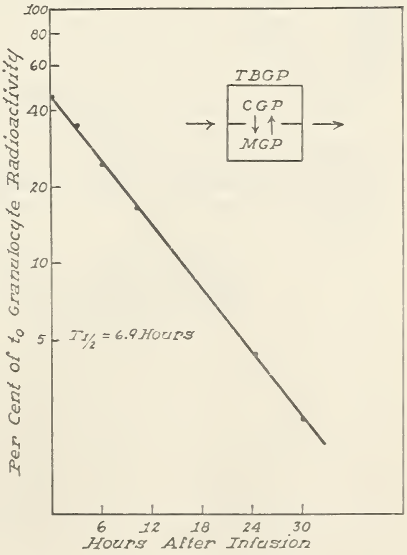


Text-figure 6.—Effect of epinephrine on recovery of labeled cells in the blood.

of cells that we could account for in the blood to an average of 83%. In several subjects who ran particularly hard we actually accounted for 95–100% of the infused cells. This has provided additional evidence that we can estimate the size of the total blood-granulocyte pool with DFP-labeled granulocytes and the dilution principle, and that there is a marginal pool of cells which apparently is in rapid equilibrium with cells circulating freely in the blood.

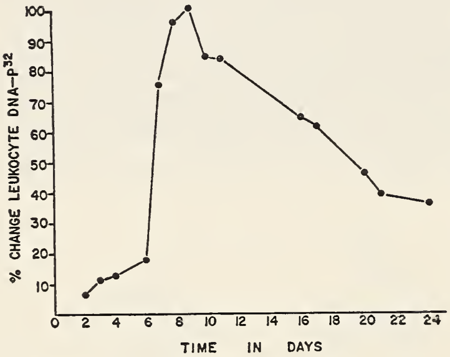
In short, we think that with this method we can measure the size of the total blood-granulocyte pool (TBGP) and the distribution of cells in circulating and marginal sites, and can follow the blood-granulocyte disappearance rate. This rate has a half disappearance time (T½) of about 6 or 7 hours in normal individuals (text-fig. 7). This means that the total mass of neutrophils in the blood is turning over or being completely replaced between 2 and 3 times a day. This is a rapidly turning over cell system as compared with the erythrocyte system which turns over only once every 120 days. The measurement of granulocyte turnover rate gives the production and destruction rate of blood granulocytes if the subject is in a steady state. Since blood granulocytes come from the marrow, we thus have a measurement of effective marrow granulocyte production.

As mentioned, blood granulocytes come from a storage pool in the marrow, the existence of which was actually demonstrated in 1958 by Perry, Craddock, and Lawrence (*4*). They demonstrated that by leukapheresis large numbers of cells could be removed from the blood without a resulting neutropenia, nor, in most studies, did a shift to the left occur. This demonstrated clearly that a pool of mature, segmented, polymorphonuclear neutrophils exists in the marrow. Perry and associates provided further evidence for the existence of this storage pool of cells in the marrow by



TEXT-FIGURE 7.—Semilogarithmic blood-granulocyte disappearance curve with model of TBGP.

labeling patients with radioactive phosphorus. Radioactive sodium phosphate was given to patients and then, at intervals over the next few days, blood samples were obtained, leukocyte DNA was isolated, and its radioactivity was measured (text-fig. 8). The curve in text-figure 8 shows about a 6-day lag period before a rise in leukocyte DNA radioactivity occurred. This indicates that there is about a 6-day lag period between the time that the last cell capable of incorporating radiophosphorus into its DNA, presumably the myelocyte, is labeled and the appearance of that cell in the blood. This is the minimal transit time from the myelocyte stage in the marrow to the blood. The peak value is reached at about 8 or 9 days and probably represents the mean time from the myelocyte stage in the marrow to the blood. Similar curves have been obtained by Cronkite's group using tritiated thymidine and autoradiography (text-fig. 9). They found a 3- to 4-day lag period before the appearance of the first labeled cell, somewhat shorter than the lag shown in text-figure 8, and a peak at about 6 days, again somewhat earlier than in the studies in which radiophosphorus was used. But clearly these data are in good agreement that there is a storage pool of cells in the marrow which is not capable of picking up DNA-labeling agents.



TEXT-FIGURE 8.—Blood-granulocyte radioactivity curve obtained after the administration of radiophosphorus. Figure 1 in (*4*).

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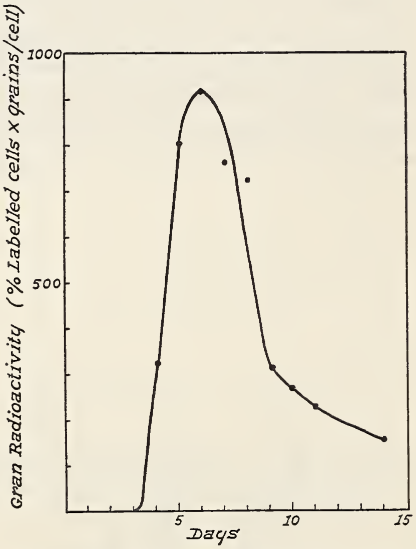

TEXT-FIGURE 9.—Blood-granulocyte radioactivity curve obtained with tritiated thymidine. Figure 3 in (*5*).

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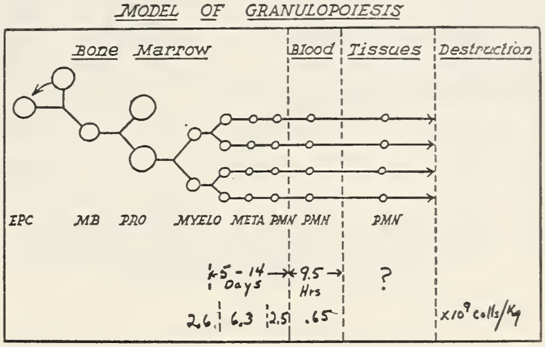
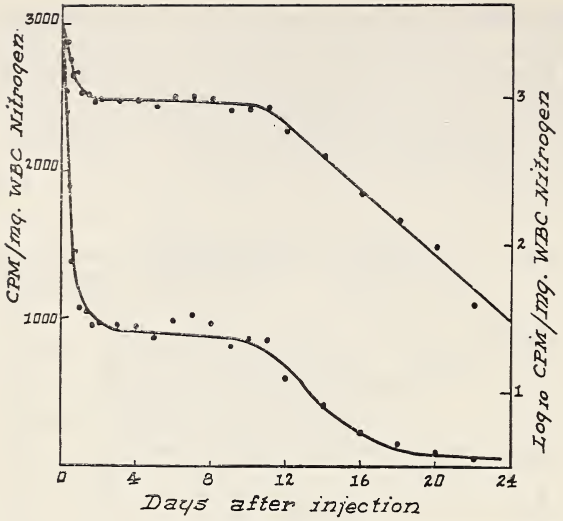
Donohue and his group in Seattle have provided the best quantitative measurement of the size of the several pools in the marrow (text-fig. 10). These figures are in good agreement with the lag times demonstrated by Perry, Craddock, and Cronkite. There are approximately 5 days' worth of postmitotic, fairly mature or maturing cells in the marrow. A rough estimate of the number of myelocytes in the marrow is also given.



TEXT-FIGURE 10.—A model of granulopoiesis.

The second technique we have used involves labeling granulocytes *in vivo* by injecting DFP intravenously. When this is done, circulating neutrophils and neutrophil precursors in the bone marrow—back to and including myelocytes—are labeled. If one follows the blood-granulocyte radioactivity after the injection of DF^{32}P, the lower curve shown in text-figure 11 results. Plotting the same data on a semilogarithmic plot gives the upper curve. The interpretation of this curve is illustrated in text-figure 12. If one draws the best line through the points in phase II, extrapolates this line back to the zero time, and then subtracts these values from the phase I values, the resulting curve is apparently identical with the *in vitro* curves. That is, the phase I values minus the phase II extrapolated values give a single exponential disappearance rate of cells with a half-time of about 7 hours. We think this provides additional evidence that taking cells out of the body, labeling them *in vitro*, and giving them back to patients do not damage them, since the T½ is the same whether they are labeled outside the body or within the circulation. From autoradiographic studies, it is clear that cells in the circulation are labeled with about 3 times as much label per cell as the precursor cells in the bone marrow. As these heavily labeled blood cells leave the blood, they are replaced by less heavily labeled cells from the bone marrow, and the phase II plateau, which in this case goes to about 11 days, represents the storage pool of such labeled cells in the marrow. In our studies in 18 normal subjects an average figure of about 11 days was obtained for the storage pool of marrow cells. This is somewhat longer than the values obtained with the DNA labeling methods. The explanation for this difference is not entirely clear, but it may be due to the differences in subjects. We have



Text-figure 11.—Arithmetic and semilogarithmic *in vivo* DFP curves. Figure 2 in (*6*).

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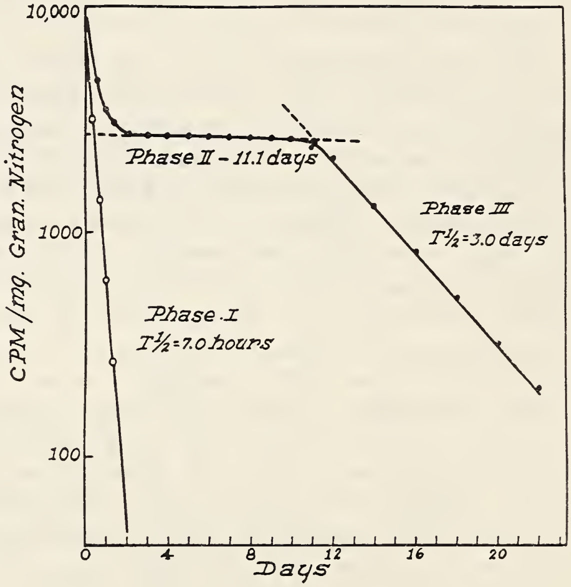

Text-figure 12.—Figure 10 in (*3*).

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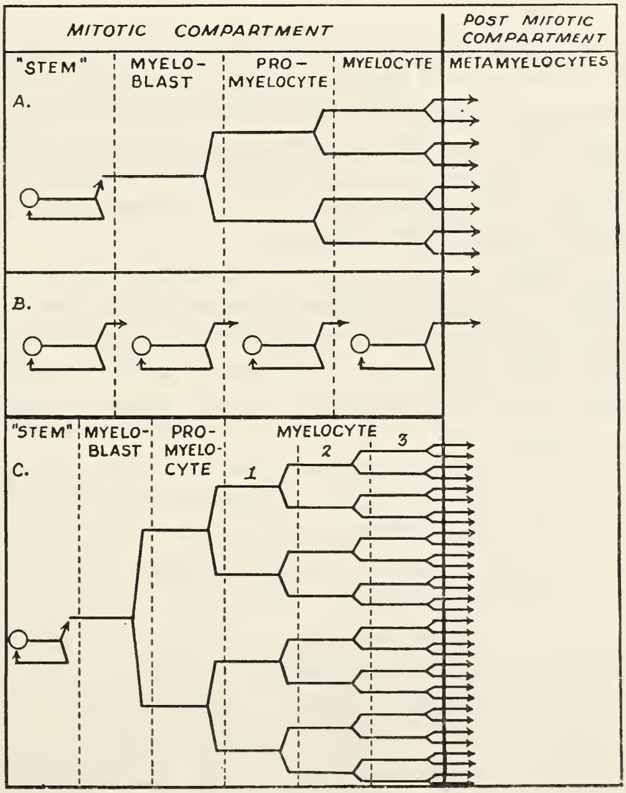
been using ambulatory, apparently healthy prisoners in our studies, while the other workers, because of the nature of their experiments, have had to use older patients, usually convalescent "hematologically normal" patients. Nevertheless, the values are not in great disagreement.

The last part of the curve or phase III in our *in vivo* curves is also of interest; the half-disappearance time of phase III is about 3 days. We think this gives information about the final dilution of the isotope in the myelocyte compartment in the bone marrow.

There are several theoretically possible cell-division schemes for marrow myeloid precursors (text-fig. 13). The one labeled "A" in this text-figure is the classical scheme in which there is a "stem" cell not morphologically identified; this cell divides and half the daughter cells become myeloblasts while half remain stem cells to perpetuate the existence of the stem-cell pool. The myeloblasts are then thought to divide, both daughter cells presumed to become promyelocytes, which divide again, and so on. The last division is presumed to be in the myelocyte stage, and the cells then mature and provide the blood and tissues with mature neutrophils. If one writes a computer model to simulate this type of production, and then compares the model curve (dashed line in text-fig. 14) with the data curve (solid line), a rather abrupt falloff in radioactivity is apparent at the end of phase II. This indicates that the classic scheme of myelocyte proliferation is not correct. Therefore, we have suggested that another scheme such as B or C in text-figure 13 describes the process better. In model B (text-fig. 13) all the cells, up to and including the myelocyte, may



TEXT-FIGURE 13.—Several models of granulocytopoiesis. Figure 6 in (7).

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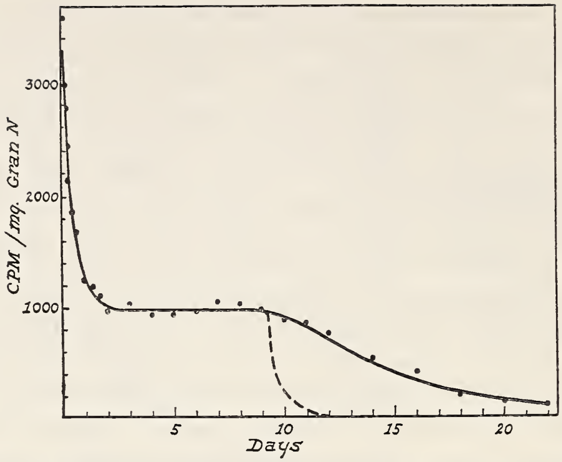
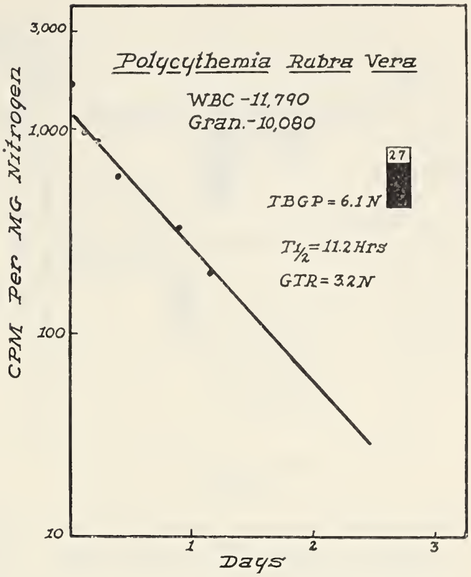


Text-figure 14.—A comparison of an *in vivo* blood-granulocyte radioactivity curve and a computer model curve generated with use of the scheme illustrated in text-figure 13A.

act as stem cells. That is, they may divide and half their progeny progress, while half remain cells of the same morphologic type, to divide again. Alternatively one can postulate a scheme such as that shown in C, in which there must be at least 3, or better 4, divisions in the myelocyte population to match the *in vivo* DF^{32}P data curves. This then is the status of our understanding of the proliferation of cells in the bone marrow, as derived from studies using the DFP technique.

Next I would like to describe some kinetic studies we have carried out in patients with various diseases. There are several ways to study granulocyte kinetics in pathologic situations. The obvious one, of course, is to study patients with disease; however, it is sometimes more productive to study a good model system, whether in an animal disease or some situation simulating disease.

In text-figure 15 the results of an *in vitro* ^{32}DFP study on a patient with polycythemia vera are shown. In patients with polycythemia vera and granulocytosis the blood-pool sizes are increased. However, in patients with polycythemia vera, and only in polycythemia vera, the size of the total blood-granulocyte pool is often increased out of proportion to the degree of elevation in blood-granulocyte count. In other words, there is a greater increase in MGP (marginated cells) than in CGP (circulating cells). This is especially true in the polycythemia patients who have granulocyte counts of 20,000 or 30,000. In patients with polycythemia vera the T½'s have been normal or somewhat long. The net effect of the large pool size and the normal or slightly prolonged T½ has been an increase in granulocyte turnover rate (GTR); that is, an increase in production and destruction rate of granulocytes. Actually the highest granulocyte pro-

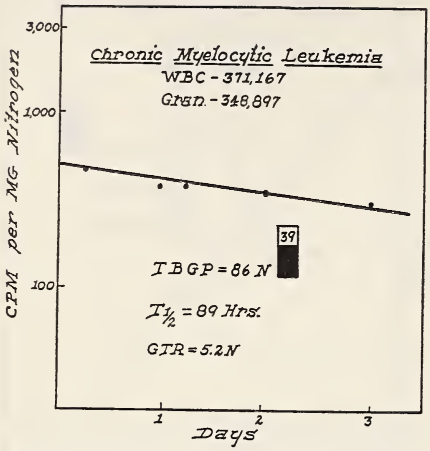


TEXT-FIGURE 15.—An *in vitro* study on a patient with polycythemia vera.

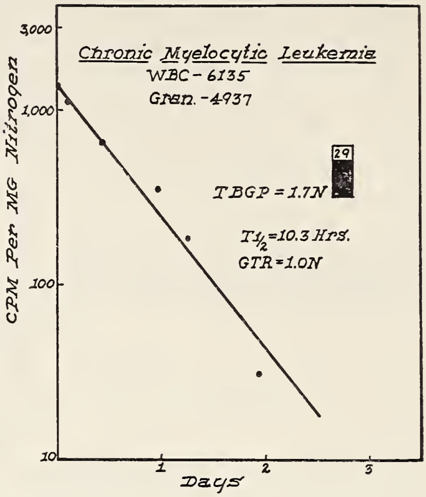
duction and destruction rates we have found have been in this disorder, running up as high as 12 or 13 times normal.

In text-figure 16 the results of an *in vitro* DF^{32}P study carried out on a patient with chronic myelocytic leukemia in relapse are shown. In this subject with a high blood granulocyte count, a tremendously high blood granulocyte pool size was found, 86 times normal. Also the T½ was very long—89 hours as compared to a normal of about 7 hours. Dr. Perry, we, and others have carried out studies attempting to find out why the T½ is long in this disorder. My opinion is that probably the long T½ reflects the fact that immature cells, myelocytes and metamyelocytes, are in the blood in large numbers, that they are labeled by this technique, and that they do not leave the blood as readily as more mature forms. There is some ancillary evidence for this in that Dr. Perry has shown the cells may recycle from the blood to the bone marrow and possibly to the spleen and has suggested that this may account for the long T½ in these individuals. In any case, when chronic myelocytic leukemia is treated by any means and the granulocyte count returned to normal, the pool sizes also return toward and finally to normal and the kinetic parameters also become normal (text-fig. 17).

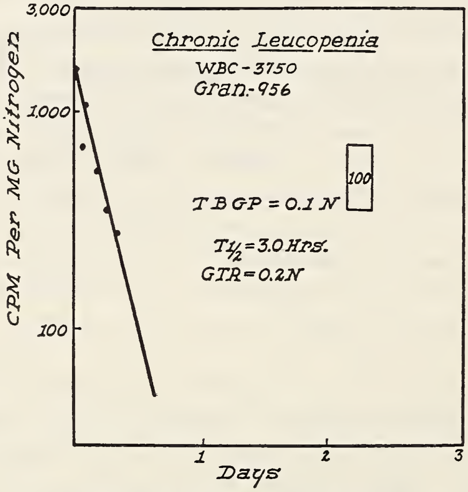
At the present time we are studying patients with neutropenias of various types. This is extremely difficult because in patients with only a thousand or so granulocytes in the blood, it is rather difficult to obtain a cell button and accurately measure radioactivity. Nevertheless, there seem to be two types of neutropenic patients. Text-figure 18 shows the results of a study on a patient whose granulocyte count and pool size are low; his T½ is at the lower end of, or slightly below, normal. This patient had been on chemotherapy and had a neutropenia due to depression of



Text-figure 16.—An *in vitro* study on a patient in relapse with chronic myelocytic leukemia.

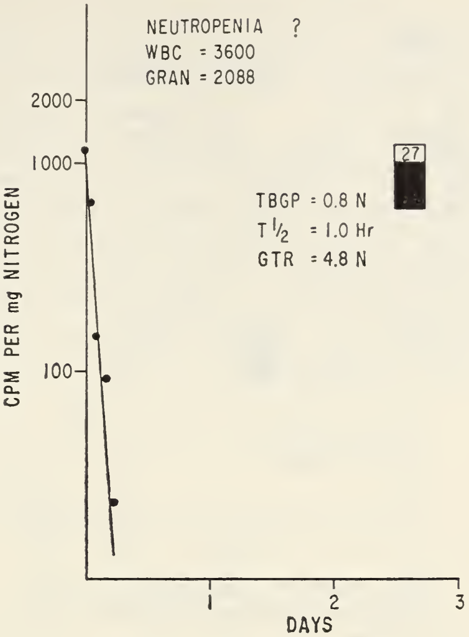


Text-figure 17.—An *in vitro* study on a patient in remission with chronic myelocytic leukemia.



Text-figure 18.—Results of an *in vitro* study on a patient with neutropenia.

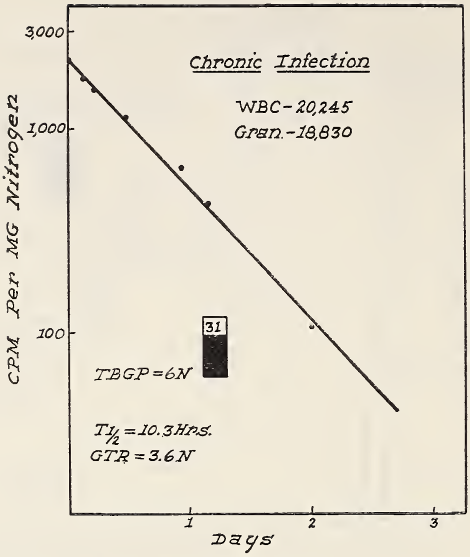
the marrow. Text-figure 19 shows the results of a study on a patient who had a low-normal granulocyte count and a blood-granulocyte pool within the normal range. However, the T½ was about 1 hour. This illustrates a group of patients who have neutropenia because of rapid granulocyte utilization and destruction rates. Even with this low count and pool size, the production and destruction rate (GTR) calculated in this patient is about 5 times normal.



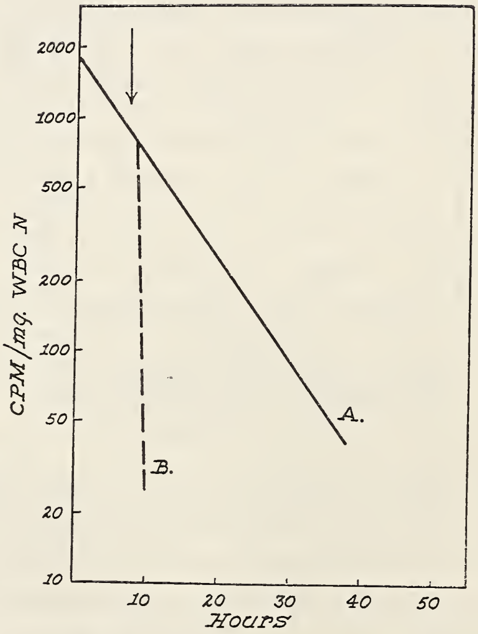
TEXT-FIGURE 19.—Results of another *in vitro* study on a patient with neutropenia.

Text-figure 20 shows the results of a study on a patient with a chronic infection, empyema. His granulocyte count and TBGP were increased. I was astounded, when we first did these studies, to find that the T½ in such patients was long. We have done a number of these studies in similar patients with chronic infection and it seems clear that this is true in all of them. They have a high normal or somewhat elevated T½. It seems that these patients provide cells to fight their infection by dumping large numbers of cells from the marrow reserve into the blood, rather than by zipping them rapidly through the blood to the site where they are needed. Because I could not believe that this was the situation in acute infection, we tried hard to study patients when they first came to the hospital, but even when we did that, we found a rather long T½.

To try to solve this problem more effectively we used a model system of the type illustrated in text-figure 21. Here is an *in vitro* curve similar to the curves that we have been discussing (solid line). If we transfuse labeled blood into a patient, establish the curve, and then perturb the system in various ways, we can get information from the effect of this disturbance on the blood granulocyte radioactivity curve. For example, if the patient is given epinephrine or made to exercise at the point indicated by the arrow, the granulocyte count becomes higher, but the curve continues its single exponential falloff (solid line A). Such a response provides evidence that



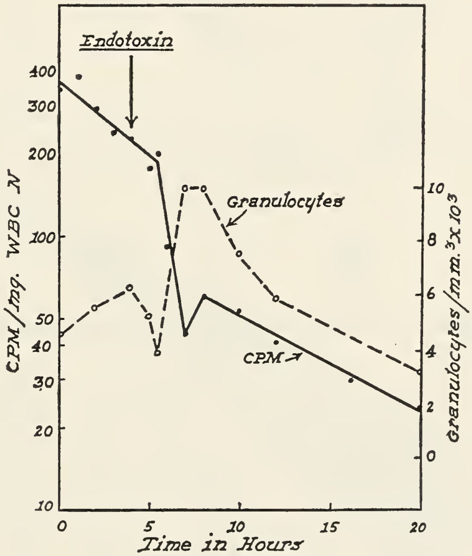
TEXT-FIGURE 20.—Results of an *in vitro* study on a patient with chronic infection.



TEXT-FIGURE 21.—A model of *in vitro* blood granulocyte radioactivity curves.

this granulocytosis is not the result of an inflow of unlabeled cells into the blood from the bone marrow. If an inflow of unlabeled cells from the bone marrow into the blood occurred, one would expect the type of curve illustrated by the dashed line B.

As a possible model for infection we studied the effect of endotoxin administration (text-fig. 22) by labeling the patient's blood, following the curve, administering endotoxin, and noting the changes in blood granulocyte radioactivity (solid line) and blood granulocyte count. About 2 hours after receiving endotoxin, the patient's granulocyte count increased from 3,000 to about 9,000 and a sharp break in his granulocyte specific-activity curve occurred, clearly indicating that unlabeled cells were entering the blood from the bone marrow to produce this granulocytosis.



TEXT-FIGURE 22.—Effect of endotoxin on blood granulocyte radioactivity curve. Figure 2 in (8).

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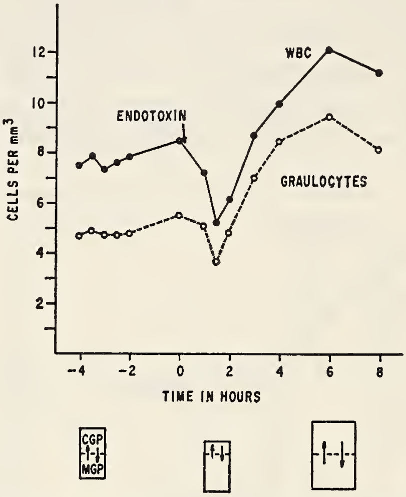
We did further studies with endotoxin, again hoping that this was a good model of the events that occur in acute infection. We gave subjects endotoxin after establishing their blood-granulocyte level (text-fig. 23). About 1–2 hours after endotoxin was given, there was a drop in blood-granulocyte count, then the count increased, reaching peak values in 5 or 6 hours in most instances. We measured the pool size and distribution of granulocytes under control conditions, in a group of subjects, as illustrated by the box on the left in text-figure 23. We then gave them endotoxin, restudied them at the low point of their neutrophil count, and demonstrated that their pool sizes were not changed, but that increased numbers of cells were present in the marginal pool, suggesting that endotoxin produced a shift of cells to marginal sites. As the granulocytosis reached a peak, we again measured pool size and found a substantial increase in size of the total blood-granulocyte pool. All this led us to postulate that after endotoxin administration there was a transient drop in count which was just an intravascular shift of cells; then the marrow was somehow stimulated to release large numbers of cells, the blood pool size increased, and the count went up.



Text-figure 23.—Effect of endotoxin on pool size and blood-granulocyte radioactivity curve. Figure 4 in (*9*).

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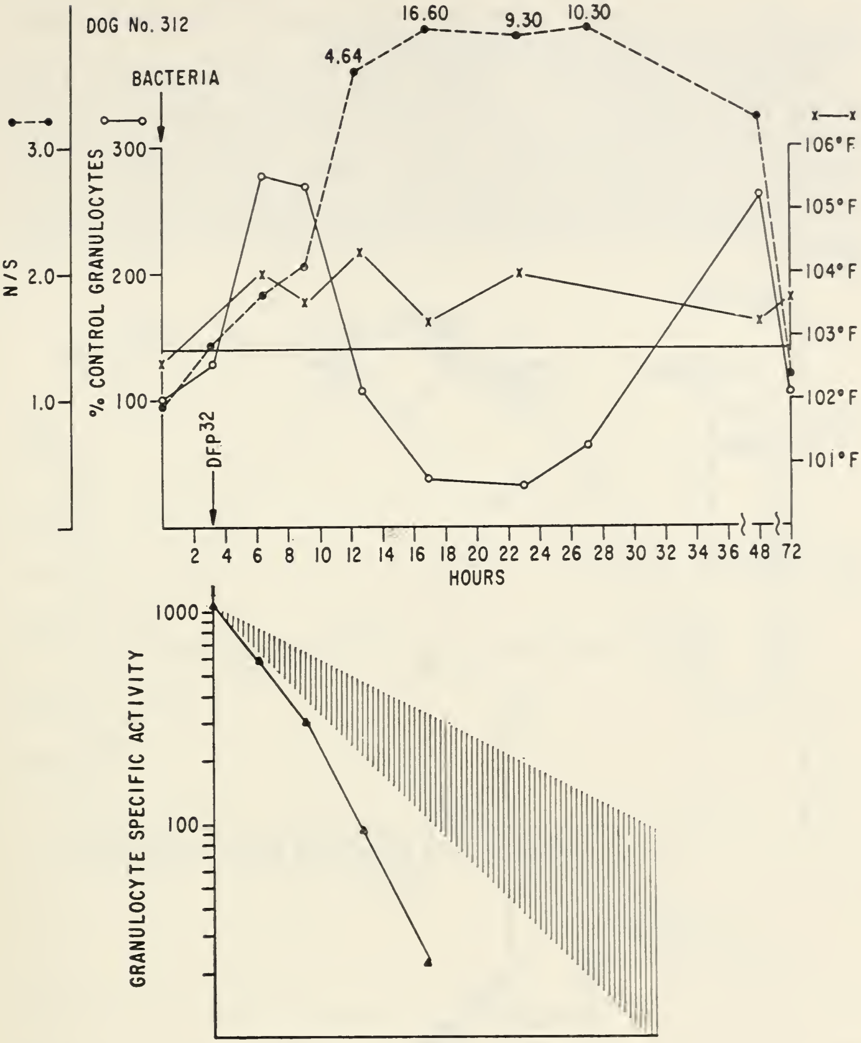
Text-figure 24 illustrates a study by Dr. Marsh and co-workers who have recently produced pneumococcal pneumonia in dogs by bronchoscoping them and injecting large numbers of pneumococci into the lungs. They followed the blood-granulocyte count (open circles) and the fever curve (X's) and they conducted DFP studies of the *in vitro* type at several points of time during the induced infection. After pneumonia was induced in dogs, the temperature usually rose within 2–4 hours, reaching a peak at about 12 hours. At about 3–4 hours, the blood-granulocyte count started up; usually it peaked at about 12 hours and then came down. The granulocyte curve in this animal (text-fig. 24) was unusual, as I will mention later. The other curve (dashed line) is the band-to-seg ratio, a measure of the shift to the left in differential count. A shift to the left started considerably after the granulocyte count began to rise and in this dog it was massive.

As already mentioned, kinetic studies were carried out at several stages during the infection: during the early stage, about 3 hours after injection of pneumococci into the lung; at 12–13 hours, when the infection was well established, and during the recovery phase. The lower portion of text-figure 24 shows that during the development of the acute infection, when the granulocyte count was just starting up, there seemed to be a shortening



Text-figure 24.—An *in vitro* DFP study carried out during the early hours of infection in a dog. Figure 1 in (*10*).

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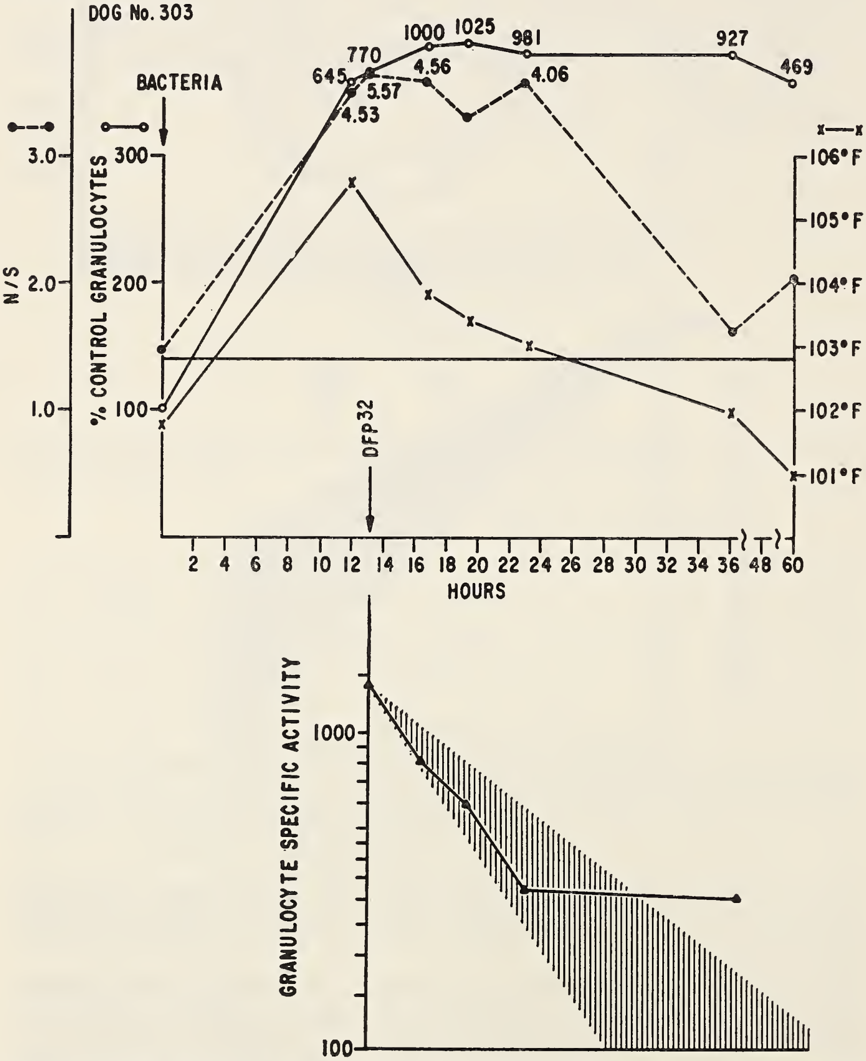
of T½ as compared to the normal range. The curve dropped rapidly as increased numbers of unlabeled cells were pulled out of the bone marrow into the blood.

The fascinating thing about this study to me is that it clearly demonstrates that a previously healthy animal, with a massive infection, can develop a severe neutropenia (open circles). I never believed this occurred in a previously healthy animal. We have all seen neutropenia in chronic alcoholics, but I have presumed this reflected an inadequate response in a patient with poor granulocyte reserves. However, this study demonstrates that, with a severe enough infection, a normal animal can indeed exhaust his granulocyte reserves and become neutropenic.

Text-figure 25 illustrates a similar type of study except that it was carried out at about 12 or 13 hours after the start of infection; however, the T½ is in the normal range. This is the type of study result we have found in all our clinical patients, and for a simple reason: We have never been able to study them until their disease is well along in its course, the patients usually being admitted 8 or 10 hours after the symptoms have begun.



Text-figure 25.—Results of an *in vitro* study carried out 13 hours after the induction of infection in a dog. Figure 2 in (*10*).

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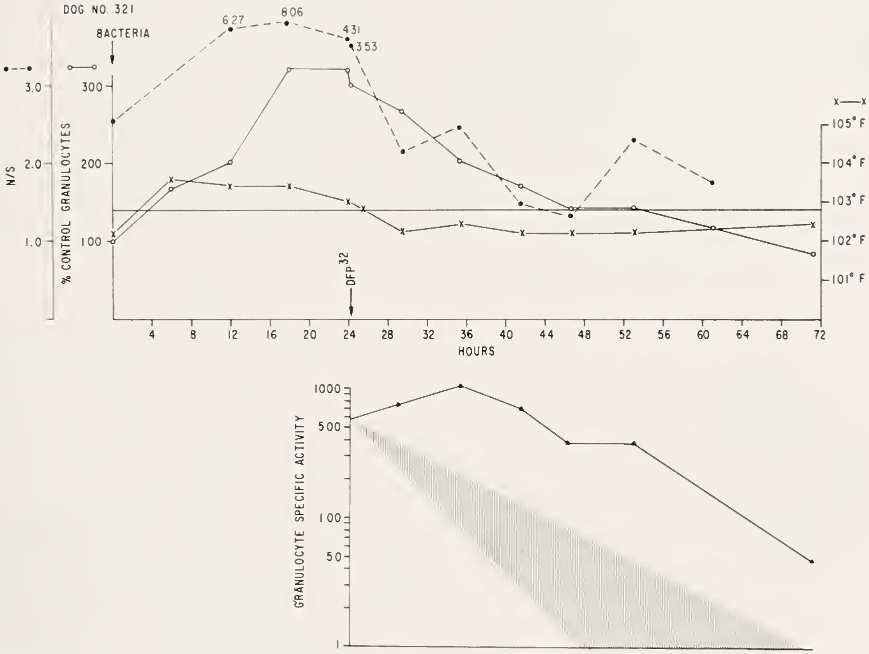
Results of a study done at 24 hours after onset of infection, during the recovery stage, are shown in text-figure 26. By this time, the granulocyte count has started down and the radioactivity values are actually above the normal range, which means that the inflow of marrow cells into the blood has been decreased or shut off. Interestingly, these studies give us some idea of the regulation of granulocyte release from the marrow. Clearly, when the animal returns toward normal, his blood-granulocyte count falls, at least in part, by shutting off inflow of cells from the marrow to the blood.



TEXT-FIGURE 26.—Results of an *in vitro* ^{32}DFP study carried out in a dog 24 hours after induction of infection. Figure 3 in (*10*).

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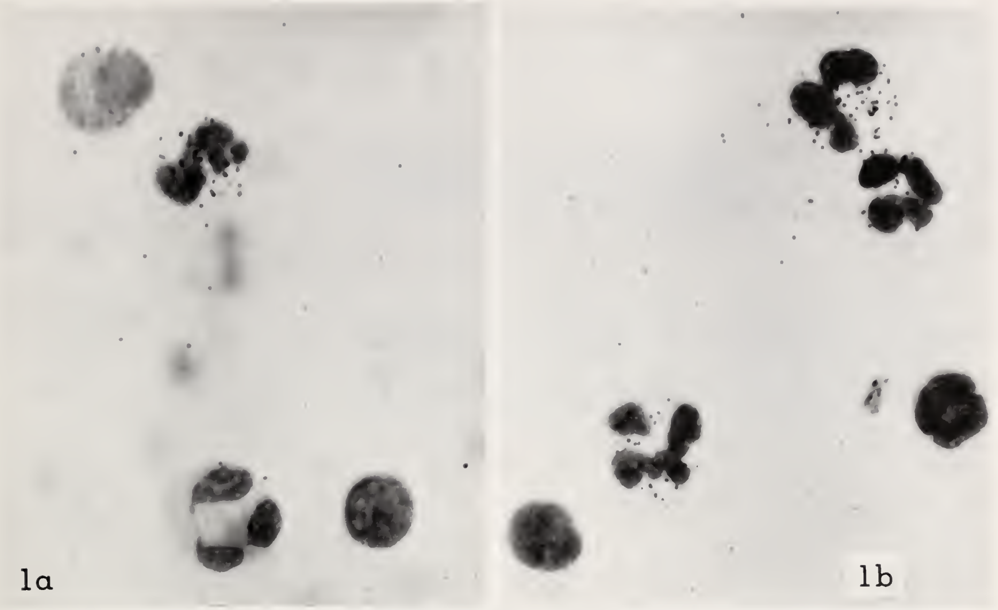

FIGURES 1A and 1B.—Autoradiographs of tritiated DFP-labeled normal leukocytes. Figure 2 in (*1*).
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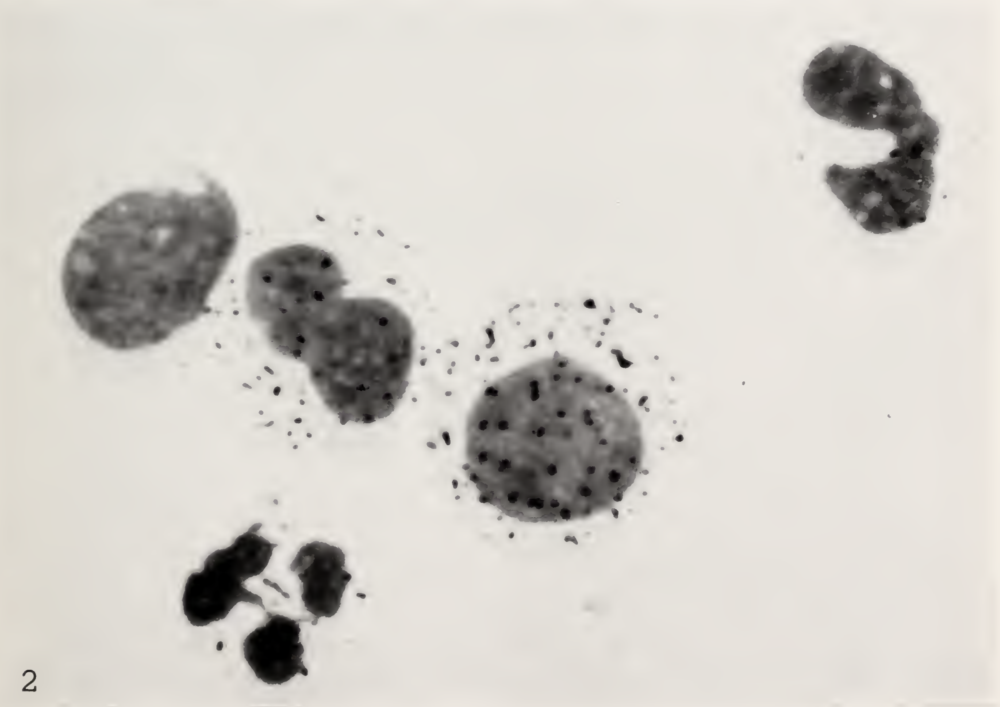

FIGURE 2.—Autoradiograph of tritiated-labeled chronic myelocytic leukemia leukocytes. Figure 1 in a paper to be published in Proceedings of International Conference on Leukemia-Lymphoma, edited by Chris J. D. Zara Fonetis. Reprinted by permission of Lea & Febiger, Publishers.

The Response of HeLa Cells to Irradiation and Hydrocortisone[1]

G. M. Kollmorgen, *Cancer Section, Oklahoma Medical Research Foundation and Department of Radiology, University of Oklahoma School of Medicine, Oklahoma City, Oklahoma 73104*

The effect of hydrocortisone on mammalian cells depends on a number of variables, including cell type, conditions of growth, and concentration of the drug. Cell killing has definitely been implied in the treatment of chronic lymphatic leukemia, as evidenced by a marked shrinkage of enlarged lymph nodes, liver, and spleen (*1*). Inhibition of growth is also indicated, since regrowth of lymphoid tumor masses occurs within days or weeks if the hormone is withheld. On the other hand, cortisol induces an absolute granulocytosis by decreasing the rate of egress of cells from the total blood granulocyte pool as well as by increasing the influx of cells from the bone marrow (*2*). Bullough and Laurence (*3*) stress the indirect action of hydrocortisone which they say tends to prolong the mitotic depression induced by adrenalin and tissue-specific chalones. They postulate that the role of hydrocortisone may be to reduce the rate of adrenalin loss. Bruce and associates (*4*) have reported that large doses of hydrocortisone produce little effect on survival of either normal hematopoietic or lymphoma colony-forming cells *in vivo.* Even though cell survival appears normal, the growth rate may be reduced. Foley and Lazarus (*5*) report that the ID50 dose of hydrocortisone for CCRF-CEM cells *in vitro* varies from 100–500 μg/ml and for other mammalian cells from 10–100 μg/ml. However, this endpoint does not distinguish cell killing (lack of reproductive integrity) from a reduced rate of movement through the generation cycle. The present investigation attempts to determine the effect of hydrocortisone on *a*) cell survival (*i.e.*, percent of the population retained in the proliferative pool) and *b*) the age-dependent response of cells in the generation cycle.

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[1] This investigation was supported in part by Public Health Service research grant FR 05538 from the Division of Research Facilities and Resources.

PROCEDURE

Cells were grown in Eagle's minimal essential medium (MEM) containing 10% calf serum, 50 U/ml of penicillin, and 50 μg/ml each of streptomycin and kanamycin. Cells were grown at 37°C and the *p*H was maintained at about 7.2 with 5% CO_2 in air. Stock cultures were grown in Roux bottles and then transferred to T-25 flasks (Falcon plastic) after EDTA trypsinization (0.7 mM EDTA and 0.025% trypsin) for 10 minutes. All cell counts were done on a Coulter counter (model B) after calibration with a hemacytometer.

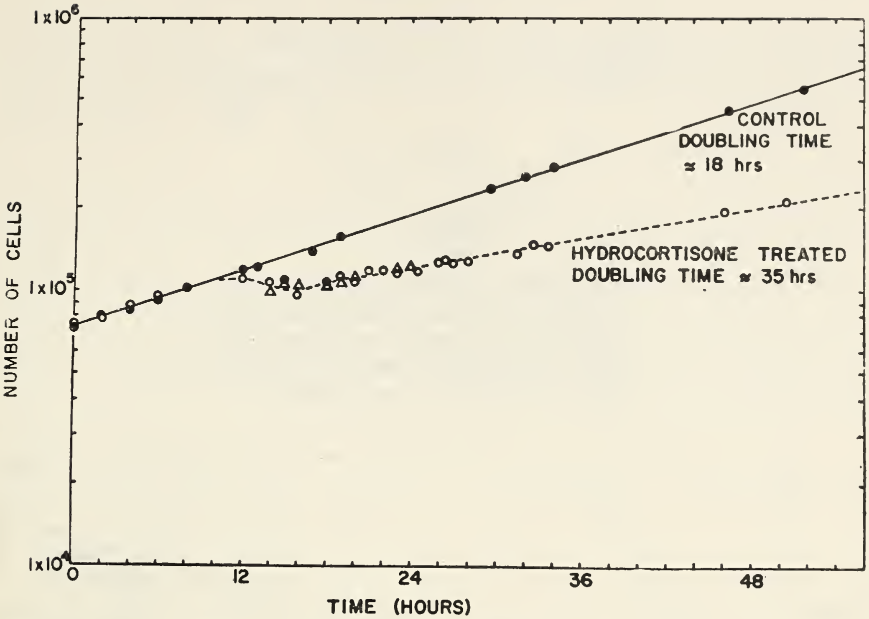
Cells were labeled with ^{3}H-thymidine at a concentration of 0.1 μCi/ml of medium (specific activity of 2.0 Ci/mmole). Pulse exposures lasted for 15 minutes. Cells were fixed with 3 parts methanol and 1 part glacial acetic acid for 15 minutes, then air-dried. Autoradiographs were prepared using Kodak NTB liquid emulsion, and cells were stained with Giemsa.

Cells were maintained at 37°C during irradiation with ^{60}Co γ-rays. The exposure rate was 39 R/minute, half-value layer of 12.5 mm lead, with a rad/R ratio of 0.97. All synchronous cultures were given 312 rads. Asynchronous populations were given 200–900 rads. Survival after irradiation was measured by counting clones containing 50 or more cells after 12 days.

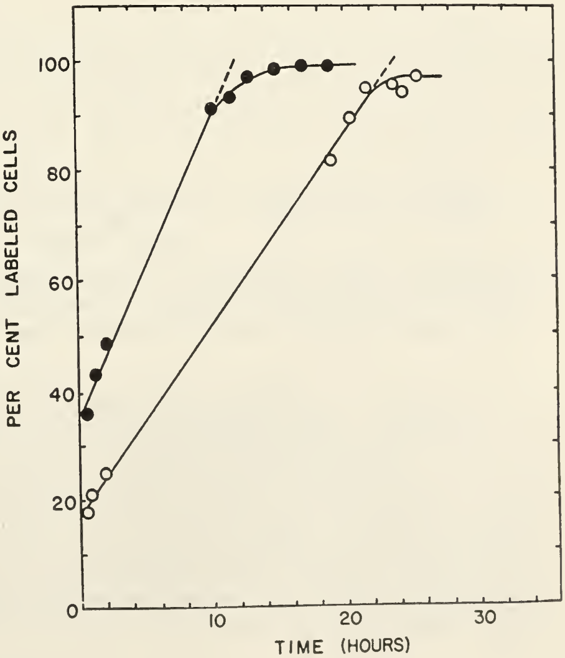
RESULTS AND DISCUSSION

HeLa Chessen cells have a doubling time of 18.0 ± 1.0 hours (text-fig. 1), and continuous labeling with ^{3}H-thymidine indicates that all cells are in the proliferative pool (text-fig. 2). The initial labeling index is about 37%, and the rate of movement is such that virtually all cells are labeled in 14 hours. Using percent-labeled mitoses curves and generation-cycle analysis as described by Kollmorgen, Trucco, and Sacher (*6*), the distribution of cells in each phase of the cycle as well as the duration of each phase is illustrated in text-figure 3.

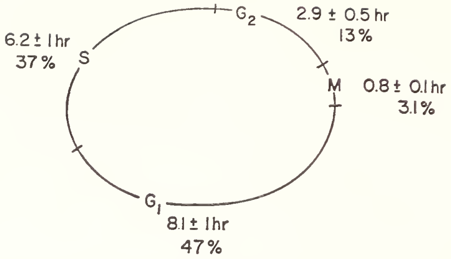
When hydrocortisone (1.7 μg/ml) is added to exponentially distributed cells in log growth, a new pattern of growth begins to emerge after 10–12 hours (text-fig. 1). This pattern is characterized by a transitional state lasting for about 6 hours, and then a new doubling time of about 35 hours is maintained thereafter. The fact that the rate of increase in cell numbers is not disturbed for 10–12 hours after the addition of hydrocortisone would indicate either a lag period of this duration or that cells in M, G_2, and S are not affected, at least during the first posttreatment cycle. Cells grown in medium containing hydrocortisone have maintained a 35-hour doubling time for more than 3 months (about 30 passages). When these cells are grown in control medium, they return to an 18-hour doubling time after about 12 hours (text-fig. 4).



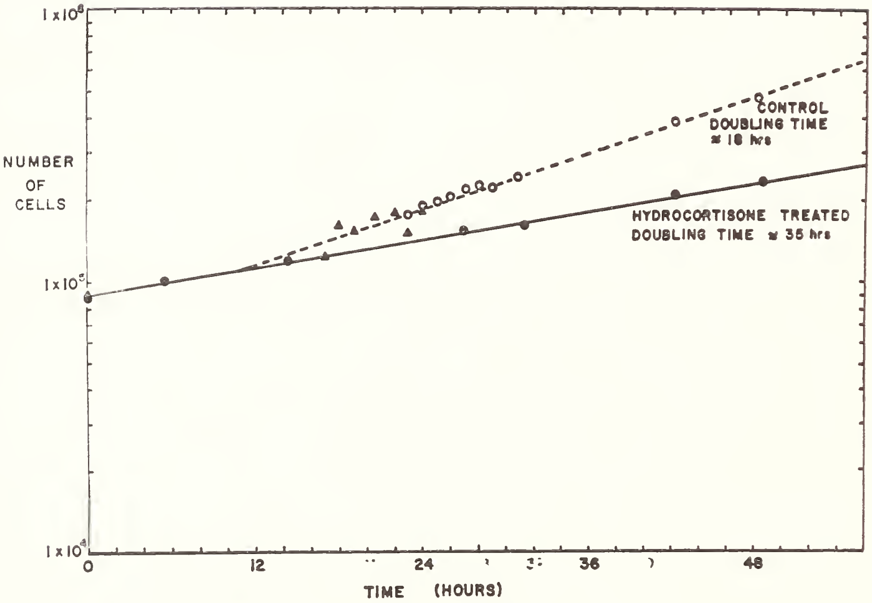
TEXT-FIGURE 1.—The total number of HeLa Chessen cells is plotted against time: ●——●, cells grown in control medium; ○——○, cells grown in medium containing hydrocortisone, 1.7 μg/ml of medium; △——△, cells grown in medium containing hydrocortisone, 0.17 μg/ml. Hydrocortisone was added at time 0.



TEXT-FIGURE 2.—The percent of labeled HeLa Chessen cells after continuous exposure to ^{3}H-thymidine (0.1 μCi/ml of medium) is plotted against time: ●——●, cells grown in control medium; ○——○, cells grown continuously in medium containing hydrocortisone, 1.7 μg/ml of medium.



TEXT-FIGURE 3.—The distribution of HeLa Chessen cells in the different phases of the generation cycle, as well as the duration of each phase when HeLa Chessen cells are grown in control medium.



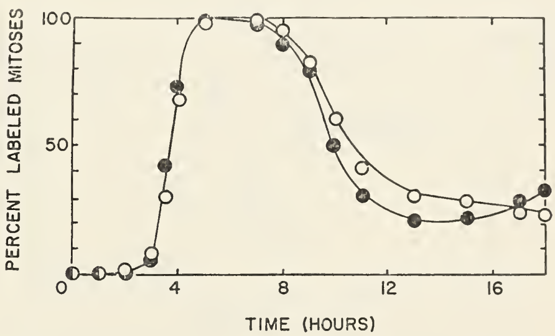
TEXT-FIGURE 4.—The total number of HeLa Chessen cells is plotted against time: ●——●, cells maintained in medium containing hydrocortisone, 1.7 μg/ml of medium; ○——○, and ▲——▲, cells returned to control medium at time 0.

Cells grown in medium containing hydrocortisone have about 94% of the population in the proliferative pool as measured by continuous labeling with ^{3}H-thymidine (text-fig. 2). The same results are obtained when clonal techniques are used to measure survival. As indicated, the initial labeling index is reduced to about 18% and these cells move through the generation cycle more slowly than control cells. Approximately 24 hours are required to achieve maximum labeling (percent labeled cells/total cells) when cells are continuously exposed to ^{3}H-thymidine. The mitotic index drops from 0.031 in control cells to 0.019 in cells grown in medium containing hydrocortisone. However, the duration of mitosis, determined by the method of Stanners and Till (*7*), is approximately 0.8 hours in both cases, where

$$T_m=\frac{m\cdot T}{0.693}$$

T_m is the time for mitosis, m is the mitotic index, and T is the generation time.

The duration of G_2 measured from percent-labeled mitoses curves is approximately 2.9 hours in both the control and the experimental group. Moreover, the duration of S is altered very little, if any, when cells are grown in medium containing hydrocortisone (text-fig. 5). Apparently, the lengthening of the generation cycle occurs entirely by the lengthening of G_1.

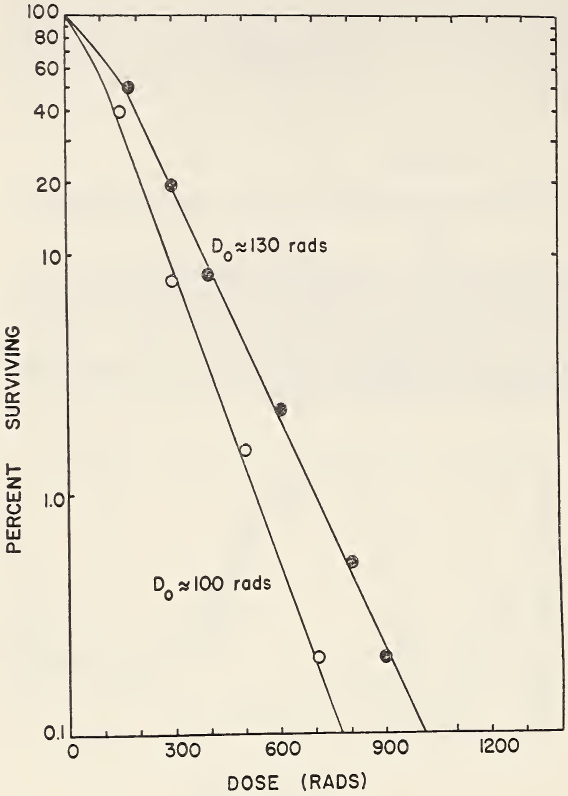


Text-figure 5.—The percent of labeled mitoses (labeled mitoses/total mitoses) in HeLa Chessen cells after ^{3}H-thymidine labeling is plotted against time: ●——●, cells grown in control medium; ○——○, cells grown continuously in medium containing hydrocortisone, 1.7 μg/ml of medium.

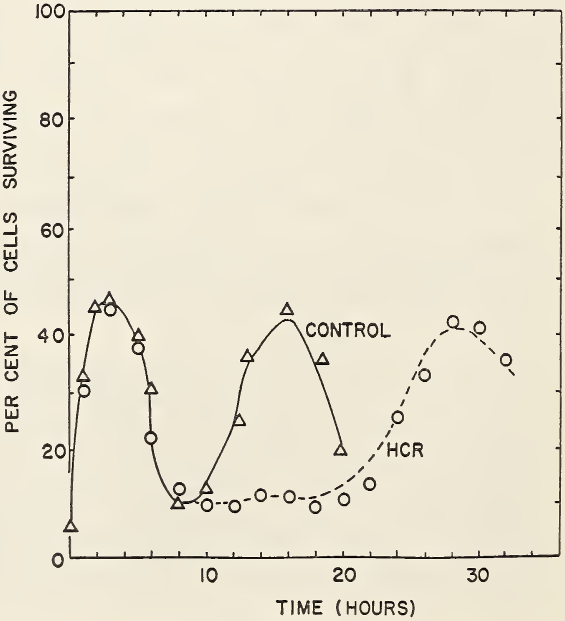
If this is true, then asynchronous cells grown in medium containing hydrocortisone may be more radiosensitive than cells grown in control medium. Several reports (*8–10*) dealing with the radiosensitivity of synchronous HeLa cells indicate that radiosensitivity increases as cells pass through G_1 and maximum radiosensitivity is attained in late G_1.

Asynchronous populations of HeLa Chessen cells grown in control medium have a D_0 value of about 130 rads and an extrapolation number of about 1.7 (text-fig. 6). When these cells are grown in similar medium containing hydrocortisone, the D_0 is reduced to about 100 rads and the extrapolation number is about 1.5. These D_0 values are significantly different at the 0.05 confidence interval.

However, the differential effect of hydrocortisone on G_1 cells is not known from these data. If cells are differentially sensitive during G_1 (*i.e.*, move more slowly through some parts of G_1), then the distribution of cells in G_1 may be inferred from percent survival curves after irradiation of synchronous cultures. Text-figure 7 shows that control cells become more radiosensitive as they pass through G_1, and that they attain maximum sensitivity in late G_1. Cells grown in medium containing hydrocortisone respond similarly for the first 10 hours, and then maximum radiosensitivity



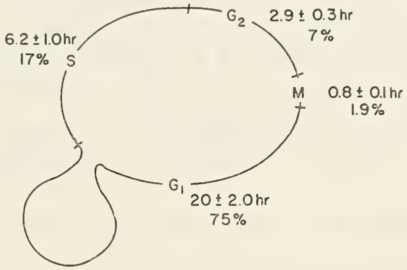
Text-figure 6.—Survival of asynchronous HeLa Chessen cells irradiated with ^{60}Co γ-rays: ●——●, cells grown in control medium; ○——○, cells grown in medium containing hydrocortisone. The standard error of each datum point is approximately equal to the size of the symbol.



Text-figure 7.—The survival of synchronous HeLa Chessen cells irradiated at different times in the cell cycle with 312 rads of ^{60}Co γ-rays is plotted against time; △——△, cells grown in control medium; ○——○, cells grown continuously in medium containing hydrocortisone, 1.7 μg/ml of medium. The standard error of each datum point is approximately equal to twice the size of the symbol.

is maintained for an additional 10 hours. The implication is that cells are temporarily blocked in late G_1.

Late G_1 cells are about 10 hours from mitosis when cells are grown in control medium. It is these cells that show the first delay in reaching mitosis after the addition of hydrocortisone. The fact that the duration of S, G_2, and M is not altered after continuous growth in the presence of hydrocortisone clearly indicates that the lesion is confined to G_1. Since a new control growth rate has been established 15 hours after the addition of hydrocortisone, the lesion must affect the sensitive G_1 cells almost immediately, and the maximum duration of cell adaptation time is about 5 hours (15 hours minus 10 hours). The rate at which cells adapt back to control medium indicates rapid cessation of the inhibitory processes when hydrocortisone is removed. The apparent distribution of these cells in the generation cycle as well as the duration of each phase is shown in text-figure 8.



TEXT-FIGURE 8.—The apparent distribution of cells in the different phases of the generation cycle as well as the duration of each phase when HeLa Chessen cells are grown continuously in medium containing hydrocortisone, 1.7 μg/ml of medium.

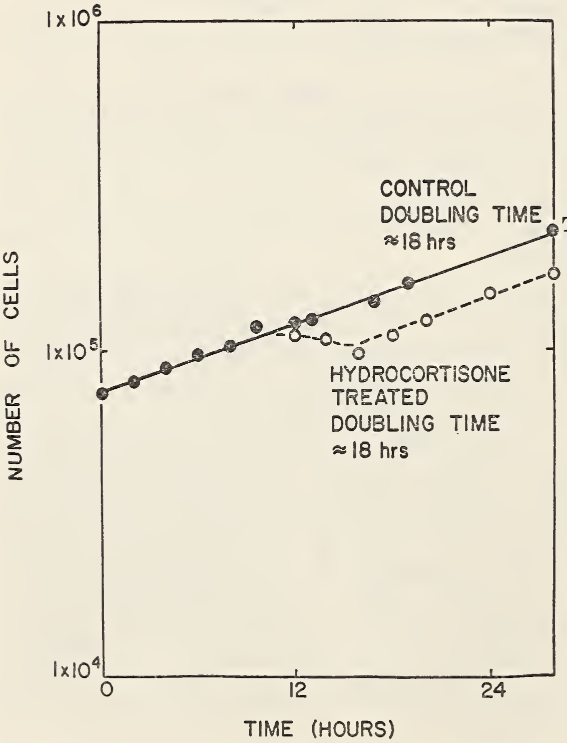
The fact that the hydrocortisone-induced kinetic lesion in HeLa Chessen is restricted to late G_1 is not surprising. Prescott (*11*) points out that regulation of cell reproduction is normally controlled by events in G_1. He suggests that regulation of proliferation in mammalian cells may be comparable to the *Tetrahymena* system where regulator proteins are required to initiate DNA synthesis.

Even though cell growth rate is decreased for HeLa Chessen cells grown in medium containing hydrocortisone and some cell killing may occur, the total protein content per cell is increased (*12*). However, the rate of protein synthesis in HeLa Chessen cells grown in medium containing hydrocortisone is similar to the rate in cells cultured in control medium. Since hydrocortisone has no effect on the movement of cells, through S, G_2, or M, it may affect some regulating mechanism(s) associated with the initiation of DNA synthesis. Dahmus and Bonner (*13*) and Sekeris and Lang (*14*) indicate that histones are involved in the process by which hydrocortisone stimulates protein synthesis in rat liver. Sluyser's (*15*) finding indicates that labeled hydrocortisone is bound mainly to rat liver histone fraction III (the fraction with the lowest lysine content) both *in vivo* and *in vitro*. Sunaga and Koide (*16*) studied the interaction of histones and DNA with cortisol in calf thymus. Labeled cortisol was bound mainly to the arginine-rich histones.

Another clone of HeLa, S_3, also grown in MEM, has virtually the same doubling time as the HeLa Chessen clone, but its response to hydrocortisone is substantially different. There appears to be a temporary delay in growth occurring 10–12 hours after the addition of hydrocortisone which is followed by the recovery of the population to again attain an 18-hour doubling time (text-fig. 9), with all cells appearing in the proliferative pool.

Both clones can be grown alternately on control medium and medium containing hydrocortisone without changing the characteristic responses described above. Both the S_3 and the Chessen clones continue to go through a kinetic transitional process when hydrocortisone is added to the medium. HeLa Chessen cells adapted to growth with hydrocortisone also go through a transition when returned to control medium. Apparently, cells which retain proliferative integrity following the addition of hydrocortisone adapt to the new environment. It is doubtful whether selection would require transitional states after the initial exposure to hydrocortisone.

The difference in the response of the two clones of HeLa cells to hydrocortisone is not readily explicable. One aspect that may be important to this difference is cellular ultrastructure. In another study (*17*), HeLa Chessen cells were shown to have a considerably more complex cytoplasmic organization than HeLa S_3 cells. Thus the lesion in the generation cycle is induced in the more morphologically complex (differentiated?)

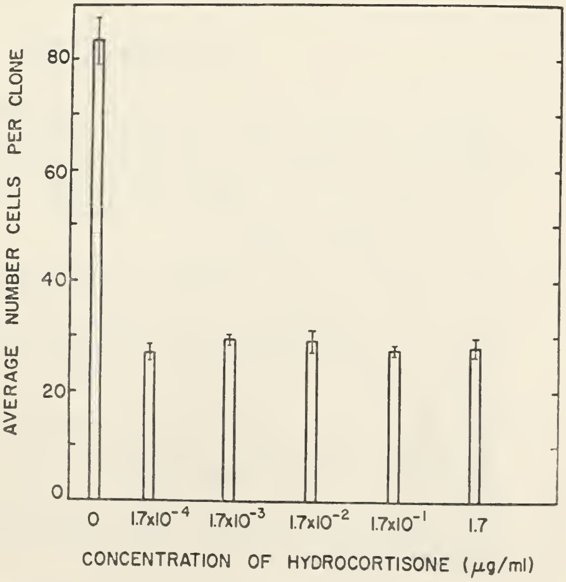


TEXT-FIGURE 9.—The total number of HeLa S_3 cells is plotted against time: ●——●, cells grown in control medium; ○——○, cells grown in medium containing hydrocortisone, 1.7 μg/ml. The standard error of each datum point is approximately equal to the size of the symbol. Hydrocortisone was added at time 0.

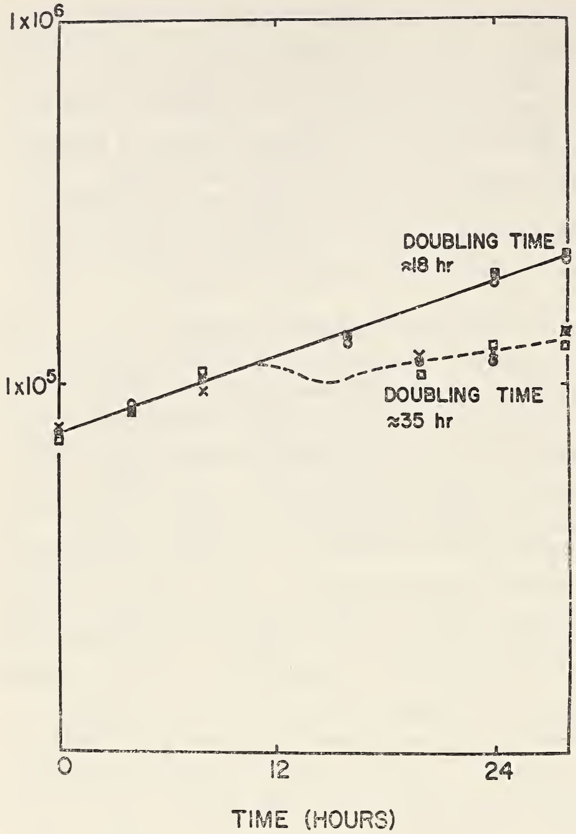
cell. This is in general agreement with Frankfurt's observations on mouse gastric epithelial cells exposed to hydrocortisone (*18*). He reports that normal cells show an inhibition in the onset of DNA synthesis for several hours, while hyperplastic cells are less sensitive and papilloma cells are completely insensitive to hydrocortisone. He suggests that this decrease in sensitivity to hydrocortisone may be one of the factors leading to accelerated growth in neoplastic cells. Several controlling mechanisms with different degrees of sensitivity may exist in different clones, or other changes like membrane alterations may be necessary to establish new rate-limiting processes.

When the initial inoculum of HeLa Chessen consisted of 4×10^2 cells/T-25 flask and cells were grown as clones (average of 6 cells/clone) before hydrocortisone was added, very small doses of hydrocortisone were required to initiate and maintain the kinetic pattern described above. Text-figure 10 illustrates that the mean clone sizes are quite similar after 3 days of growth with the use of hydrocortisone doses of 0.00017 to 1.7 μg/ml of medium. Statistical analysis indicates that neither means nor distributions of these populations are different at the 0.05 confidence interval. When the initial inoculum is increased to 4×10^5 cells/T-25 flask, and the initial clone size is 1.5 cells, the dose response of these cells to hydrocortisone is quite different. Doses of 0.017 μg/ml of medium and above initiate the response illustrated in text-figure 1, while similar doses have no effect during the following 30-hour interval (text-fig. 11).

Membrane changes following hydrocortisone have been previously reported by several workers (*19–21*). It may in fact be membrane characteristics, developed during clonal growth, that cause cells to become more sensitive to lower doses of hydrocortisone when grown as larger clones.



Text-figure 10.—The average number of HeLa Chessen cells per clone after 3 days' growth in different concentrations of hydrocortisone is plotted. The initial clone size averaged 6 cells/clone. Standard errors are indicated.



Text-figure 11.—The total number of HeLa Chessen cells grown in medium containing different concentrations of hydrocortisone is plotted against time. The average clone size when hydrocortisone was added was 1.5 cells per clone: ○——○, 0.00017 µg/ml; ■——■, 0.0017 µg/ml; x——x, 0.017 µg/ml; □——□, 0.17 µg/ml; ●——●, 1.7 µg/ml. Hydrocortisone was added at time 0.

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Studies on Cell Population Kinetics in the Regenerating Liver[1]

JACOB I. FABRIKANT,[2] *Department of Radiological Science, The Johns Hopkins University, Baltimore, Maryland 21205*

REGULATORY control mechanisms, concerned with cellular proliferation and differentiation which maintain population size and function in steady-state cell renewal systems, are poorly understood. Little is known about population level regulation, such as feedback control mechanisms influencing the degree and rate of cell turnover, and about biochemical mechanisms involved in the initiation of differentiation. While there may be a valid distinction between the proliferation and differentiation kinetics of normal cell renewal and those of tumor growth, certain important kinetic relationships involving cellular control mechanisms may obtain between conditional cell renewal and tumor cell systems. Both populations appear to have variable rates of cell turnover, are self-maintaining, and are primarily proliferative; the maintenance of cell number does not necessarily depend on a precursor cell population, and only a proportion of cells may be proliferating. Major differences exist, however. In conditional cell renewal systems, homeostatic mechanisms achieve a balance between cell population size, the rate of cell transfer from nonproliferating to proliferating compartments, and the rate of cell loss, for example, for differentiation. The fine structure of this balance is profoundly disturbed in neoplastic growth, and a better understanding of cellular regulatory mechanisms in conditional cell renewal systems may provide information on the changes in the patterns of tumor cell population kinetics leading to uncontrolled growth and, frequently, a loss of morphological or functional evidence of differentiation.

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[2] Much of this work was carried out while I was an Advanced Fellow of Academic Radiology of the James Picker Foundation on recommendation of the NAS-NRC at the Department of Biophysics, Institute of Cancer Research, Belmont, Sutton, Surrey, England. I am indebted to Professor L. F. Lamerton for his generous help, suggestions, and criticisms.

The mammalian liver is a conditional cell renewal system that ordinarily undergoes little proliferation, but may do so in response to a demand situation to compensate for a loss of cells, for example, after partial hepatectomy. The complex cellular kinetics of the controlled compensatory growth, and particularly the rapid changes in the patterns of DNA synthesis and cell division in the different cell populations, which are constantly altering such relevant kinetic parameters as proliferation rates, transit rates, and compartment sizes, have not been elucidated. The present autoradiographic study of DNA snythesis and the cell cycle in the regenerating rat liver concerns the analysis of cell population kinetics[3] (*1*, *2*) during the period of rapid cell proliferation following two-thirds partial hepatectomy. By use of techniques of *in vivo* labeling with tritiated thymidine (^{3}H-TDR), a specific DNA precursor, combined with high resolution autoradiography, a detailed investigation was made of the kinetics of cellular proliferation of the parenchymal cells, littoral cells, bile duct epithelium, and other cell populations of the resting liver and of the regenerating liver in 6- to 8-week-old male rats of the August strain during the first 64 hours after partial hepatectomy. The purpose was: 1) to examine their temporal and spatial patterns of proliferation, and 2) to evaluate the dynamic contributions of the cell populations taking part in the regenerative process leading to restoration of the hepatic tissue deficit.

PATTERN OF PARENCHYMAL CELL PROLIFERATION

The temporal pattern of labeling of parenchymal cells during the 64 hours after partial hepatectomy is illustrated in text-figure 1*a*. There was little or no DNA synthesis until 12 hours, at which time the first labeled cells were seen. This was followed by a rapid rise in the percentage labeling index to a peak of 44% at 25 hours, and then by a gradual decline to 64 hours. Labeling indexes remained elevated for a number of days thereafter, and returned to normal after 2–3 weeks when regeneration was complete. Cells in mitosis first appeared at 21 hours; the mitotic index rose rapidly to 5% at 33 hours, followed by a gradual decline to approximately 1% at 44 hours (text-fig. 1*b*). A second and smaller wave occurred between 52–60 hours. The initial wave of mitosis followed that of labeling by some 8 hours, and allowing for this interval, there were 5–10 times as many cells labeled as in division. The mitotic index remained

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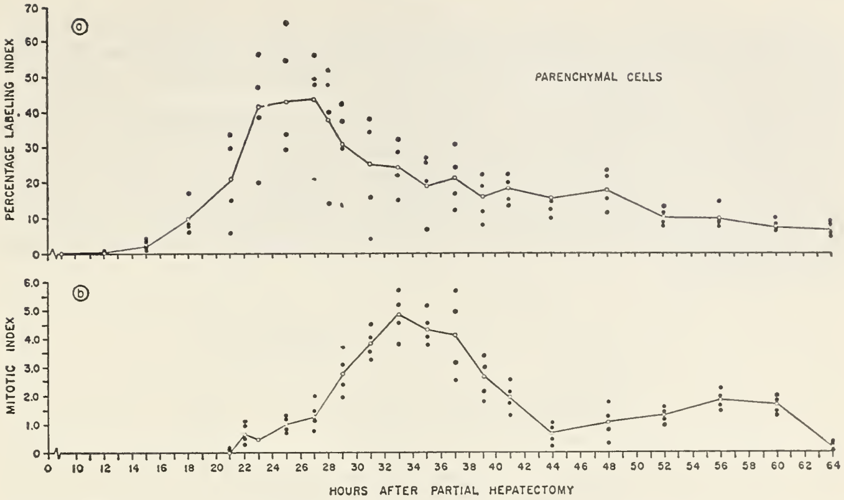
[3] The following abbreviations are used in analysis of cell population kinetics: C, cells in cell cycle; M, cells in mitosis (M period); S, cells in DNA synthesis (S period); G_1, cells in presynthetic period; G_2, cells in postsynthetic period; G_0, potentially proliferative cells; Tr, transitional and mature cells: T_c, duration of cell cycle; T_m, duration of M period; T_s, duration of S period; T_{g_1}, duration of G_1 period; T_{g_2}, duration of G_2 period; $T_{g_2+m/2}$, duration of $(G_2 + M/2)$ complex period.



Text-figure 1.—(*a*) Temporal pattern of incorporation of ^{3}H-thymidine in proliferating parenchymal cells during regeneration; (*b*) temporal pattern of parenchymal cell mitoses during regeneration. Mean values for 4 rats per interval.

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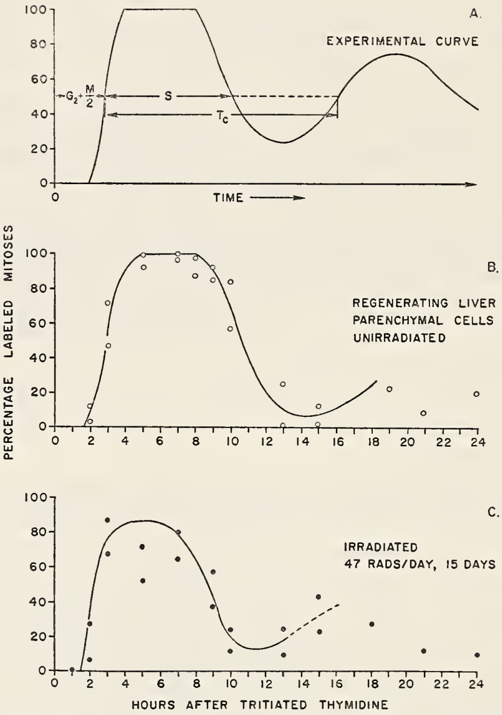
elevated for a number of days thereafter, and occasionally cells in division were observed 1 and 2 weeks later. The relevant kinetic parameters are summarized in table 1.

The percentage labeled mitoses technique for measurement of the phases of the cell cycle involves the pulse-labeling of a population of proliferating cells during DNA synthesis and follows them through the cell cycle. The cell cycle consists of at least four phases: M, G_1, S, and G_2; in the regenerating liver, the system may contain a G_0 population of potentially proliferative cells. Rats were partially hepatectomized, given injections of ^{3}H-TDR 20 hours later, and killed at frequent intervals from 1 hour to 44 hours thereafter. T_{g2}, T_m, and T_s were measured according to the method of Quastler and Sherman (*3*), in which the relationship between the percentage of mitoses labeled and time and the phases of the cell cycle is examined (text-figs. 2*A* and 2*B*). The shortest T_{g2} was 2.0 hours, and

Table 1.—Kinetic parameters of parenchymal cell proliferation

Group	DNA synthesis				Mitosis		
	Onset (hr)	Peak (hr)	Labeling index (%)	Mean rate of entry (%/hr)	Onset (hr)	Peak (hr)	Mitotic index (%)
Unirradiated	12	27	46	4	21	36	7
Irradiated	18	30–40	28	3	30	38	6

the $T_{g_2 + m/2}$ complex period was $\sim$3.0 hours. The mean T_s, measured at the 50% level of the ascending and descending limbs of the wave of labeled mitoses, was $\sim$8.0 hours. A second wave was absent, and since analysis by this method requires at least two divisions for the accurate determination of the cell cycle time (and thus, T_{g_1} as well), only T_{g_2}, T_m, and T_s could be measured with precision. Labeled mitotic figures with very low grain counts were seen 44 hours after ^{3}H-TDR, indicating that a few parenchymal cells probably divided two or more times during the first 64 hours of regeneration (*4*).



TEXT-FIGURE 2.—Percentage labeled mitoses curves. (*A*) Cell renewal system; (*B*) proliferating parenchymal cells in regenerating liver in unirradiated rats; and (*C*) after continuous irradiation. Each *point* represents 1 rat.

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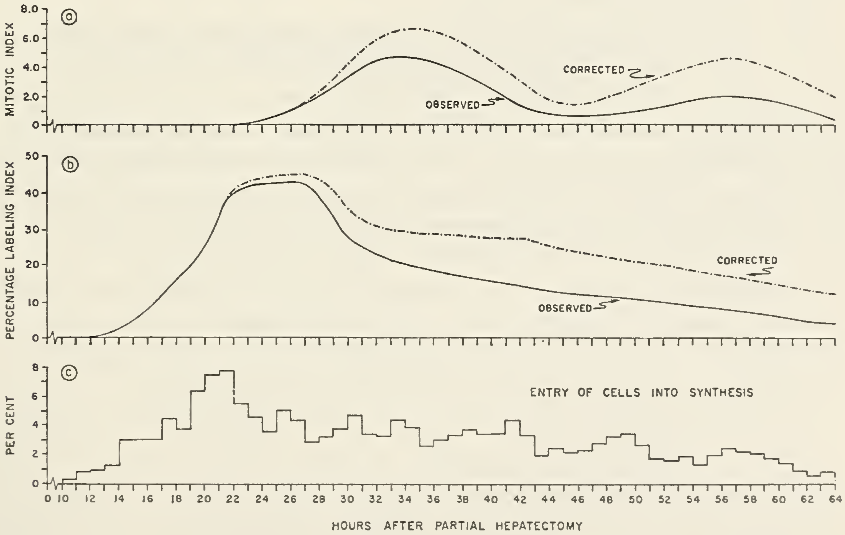
ANALYSIS OF PARENCHYMAL CELL POPULATION KINETICS

The burst of proliferative activity in the liver remnant during the first 64 hours results in a marked tissue expansion of cells with little or no cell loss. To obtain accurate information for much of the quantitative analysis of the pattern of cell proliferation, the percentage labeling and mitotic indexes must be corrected for the increasing mass of the liver to provide a measure of the total number of cells in synthesis and division in the remnant at any given time. An estimate of changes in the number of parenchymal cells at frequent intervals can be based on the temporal course of the mitotic index by use of the methods of Smith and Dendy (*5*) and Goss (*6*). This was done for the mitotic index at hourly intervals based on a curve of best fit to the means (text-fig. 3*a*). From the labeled mitoses curve data, T_m was taken to be 1.0 hours and T_s, 8.0 hours. This estimate of liver size, taking the size of the remnant after partial hepatectomy to be 1.0, is based on the hypothesis that at the end of each hour a number of cells equal to the number in mitosis during the hour is added to the cell population present at the beginning of the hour. This was determined progressively throughout the course of the regeneration, the number of cells in mitosis being calculated from the observed mitotic index and the estimated size of the liver at the beginning of the hour.

The *corrected mitotic index* is defined as the total number of parenchymal cells dividing in the liver remnant at time *t*, expressed as a fraction of the number of cells in the remnant at the time of partial hepatectomy. The *corrected labeling index* is the total number of parenchymal cells in



TEXT-FIGURE 3.—(*a*) Derivation of mitotic indexes for parenchymal cells corrected for growth of the liver remnant during regeneration, assuming $T_m=1.0$ hour; (*b*) derivation of corrected labeling indexes for parenchymal cells, assuming $T_s=8.0$ hours; (*c*) hourly rate of entry of parenchymal cells into DNA synthesis determined from corrected labeling index data. Observed curves are plotted as best fit through the means.

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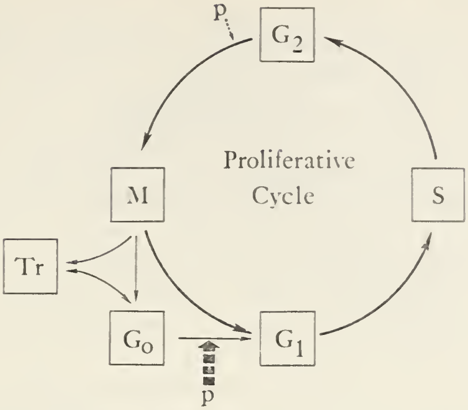
synthesis in the liver remnant at time *t*, expressed as a fraction of the number of cells in the remnant at the time of partial hepatectomy. Comparison of the changing patterns of the observed and corrected labeling and mitotic indexes during the 64 hours of regeneration indicates that the peaks of greatest synthetic and mitotic activity in the liver remnant reached higher levels and occurred later (text-figs. 3*a* and 3*b*). Thus, the total number of proliferating cells was greater than estimates obtained from uncorrected curves of the observed data. Text-figure 3*c* illustrates the rate of entry of parenchymal cells into DNA synthesis after partial hepatectomy determined from the corrected labeling index data. After a burst of synthesis in a relatively large population of hepatocytes, there was a steady passage of cells through synthesis at a rate of 3–4% per hour until 50 hours, followed by a gradual decline. The efficiency with which the constant rate was maintained for some 24 hours indicated that after the initial wave of synthesis, mechanisms were operative to control the rates of synthesis and cell division in a manner similar to that of a steady-state renewal population, though without loss of cells through migration or death. By 64 hours, the cumulative hourly corrected labeling index and corrected mitotic index both increased by a factor of ~1.7, indicating an efficient synthesis-mitosis sequence and that a large proportion of the cell deficit was replaced within 3 days.

The present studies and repeated labeling experiments have shown that all but a few parenchymal cells in the region of the central vein of the lobule became labeled during regeneration; thus, most parenchymal cells of the liver comprise the potentially proliferative cell population. It appears, therefore, that 1) most parenchymal cells in the normal liver are in a state of dormancy with respect to renewal and growth (probably, G_0), 2) in this state, the hepatocyte performs the functions of the normal liver cell but retains its proliferative capacity, and 3) when the proper stimulus arises, in response to a demand situation, such as partial hepatectomy, the cell is triggered off to enter and proceed through a cell cycle. After division, the cell may enter a state of dormancy (again, probably G_0 or prolonged G_1 phase), indistinguishable from transitional or mature functional parenchymal cells, once again until triggered off subsequently (text-fig. 4). There is evidence that a few cells entered division without synthesizing DNA, indicating that some cells were stored in the premitotic G_2 period from which, if conditions permit, they could enter mitosis. Such a G_2 store apparently has been recognized in skin epithelium (*7*) and may very well obtain for certain tumor cell populations (*8*). After partial hepatectomy, few cells or none are lost from the proliferating population during regeneration, and most parenchymal cells divide only once—and a few twice—and subsequently enter into the dormant or stored population of cells with very low turnover.



TEXT-FIGURE 4.—Scheme of the compartments of the parenchymal cell proliferative cycle as a conditional renewal system based on the general model described by Quastler (2). The cell cycle shows 4 phases: G_1, S, G_2, and M. The system contains a population of potentially proliferative G_0 cells which produce at a low rate or only on stimulation (p = partial hepatectomy) and a population of transitional or mature Tr cells.

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SPATIAL DISTRIBUTION OF PROLIFERATING PARENCHYMAL CELLS

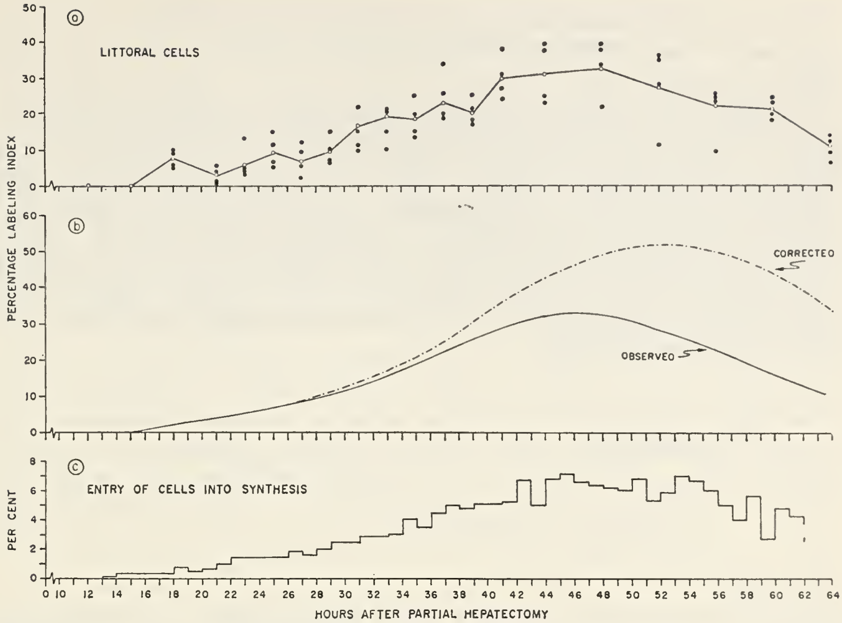
The distance between the portal triad and the central vein in the liver lobule was subdivided into thirds, thereby defining an outer, middle, and inner zone. During the first 64 hours after partial hepatectomy, all lobules in the remnant were similarly labeled and ^{3}H-TDR was available equally to all cells in synthesis. The earliest evidence of labeling of parenchymal cells occurred in the periportal area; at the peak of synthetic activity, the labeled nuclei were concentrated in the outer zone and, with time, labeling of cells extended toward the middle and inner zones and eventually became randomly distributed throughout the lobule. It appeared that during regeneration most of the outer zone cells entered synthesis and divided more than once, whereas a number of inner zone hepatocytes did not proliferate at all during regeneration. The wave of mitotic activity which followed DNA labeling exhibited the same pattern of temporal and spatial distribution within the lobule. Initially, almost all mitoses occurred in the outer zone; the rapid birth of new cells resulted in the redistribution of zonal boundaries. With subsequent cell division, the mitotic index in the middle zone increased rapidly; however, the outer half of the lobule contributed the greatest number of new cells.

Evidence linking the anatomical pattern and biochemical anatomy of the liver lobule with sinusoidal blood circulation includes both altered gradients in blood composition along the sinusoid (particularly oxygen and certain enzymes and metabolites) and changing histochemical characteristics of the zones involving glycogen and lipid metabolism manifested as diurnal variation and during digestion (*9*). It is likely that all parenchymal cells appear the same structurally, have equal functional capacity, and can adapt depending on the location of the cell in the lobule. Differences in metabolism of the three zones may help explain characteristic changes in the temporal and spatial patterns of parenchymal cell proliferation during regeneration and the utilization of available DNA precursor to proliferating cells. RNA and DNA synthesis and cell division occur in sequence beginning in the outer zone and progressing in waves toward the central zone. Peripheral zone cells divide once and a few of them twice, whereas some of the centrilobular zone cells do not proliferate at all. It therefore appears that the signal for proliferation does not affect all the parenchymal cells to the same extent. After regeneration, the degree of ploidy increases, particularly in the peripheral zone, and approaches the complex pattern characteristic of the centrilobular zone cells.

PROLIFERATION OF LITTORAL CELLS

The earliest appearance of labeled littoral cells occurred at 18 hours after partial hepatectomy (text-fig. 5*a*). There was a gradual increase in the percentage labeling index to approximately 30% at 42–50 hours, followed by a gradual decline to approximately 10% by 64 hours. The maximum mitotic indexes occurred from 48–56 hours. Labeled cells were distributed randomly throughout the liver lobule during regeneration, and mean grain counts varied widely. Since the first labeled littoral cell mitoses appeared 3 hours after ^{3}H-TDR, and since the numbers of mitoses were greatest 8–10 hours after the peak of labeling, the interval between the onset of DNA synthesis and cell division did not exceed 10 hours. Assuming $T_s =$ 7.5 hours, and corrected labeling index data (text-fig. 5*b*) indicated that the period of greatest littoral cell proliferation reached a higher level and occurred later than would appear from the observed percentage labeling index data. The rate of entry of littoral cells into synthesis during the 64 hours (text-fig. 5*c*), derived from the corrected labeling index data, slowly increased during the first 25 hours, rapidly rose to a maximum by 46 hours, and then declined gradually. Between 40 and 55 hours, the mean rate of entry was 6% per hour and by 64 hours the ratio of the number of cells which entered synthesis to the number originally present was ~1.9. Thus, by 64 hours, all but some 5% of the littoral cell population necessary to replace the cell deficit had entered synthesis.

The delay in the onset of DNA synthesis in littoral cells for some 24 hours after the onset of parenchymal cell proliferation suggested that the signal for proliferation in the littoral cells may have been in response to



Text-figure 5.—(*a*) Temporal pattern of percentage labeling indexes in proliferating littoral cells during regeneration; mean values for 4 rats per interval; (*b*) derivation of corrected labeling indexes for littoral cells, assuming $T_m = 1.0$ hour and $T_s = 8$ hours; (*c*) hourly rate of entry of littoral cells into DNA synthesis determined from corrected labeling index data.

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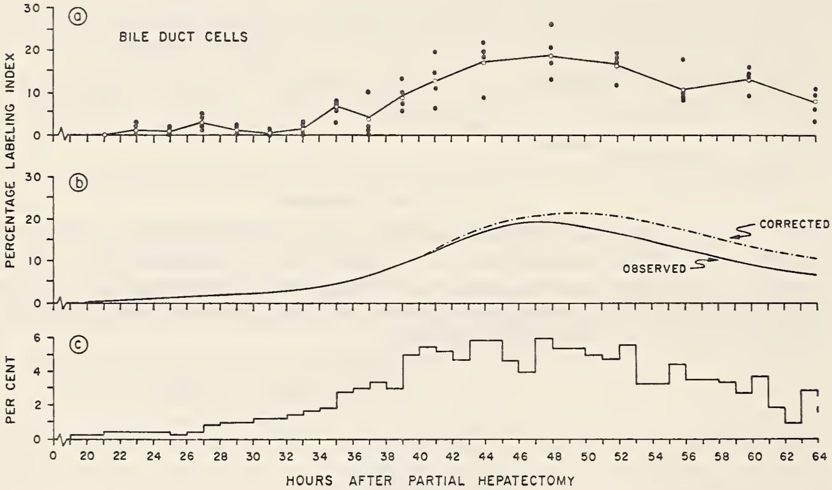
growth of the parenchymal cell population. During the first 64 hours, the mean rate of entry of new cells into synthesis was over 4% per hour, which was greater by a factor of 40 than the normal proliferation rate of the reticuloendothelial system (RES) in the resting liver. However, it should also be recognized that extensive transformation and migration of littoral cells occur during regeneration (*10*) and up to some 50% of the RES cell population in the liver remnant, particularly during the early period of regeneration, may migrate to the liver from the extrahepatic RES reserves to augment the deficiency.

PROLIFERATION OF INTERLOBULAR BILE DUCT EPITHELIUM

The first evidence of proliferative activity in the bile duct epithelium occurred at 23 hours (text-fig. 6*a*); the increase in labeling was gradual until 35 hours, after which time the percentage labeling index rose to a peak of 19% at 48 hours, and then decreased gradually. Only few mitoses were observed, the maximum numbers occurring between 44 and 64 hours.

The first labeled mitotic figures in the interlobular bile duct epithelium appeared 3 hours after ^{3}H-TDR at 48 hours. Since the interval from the onset of S to the onset of M was 10 hours, and $T_{g2}=3$ hours, then T_s was $\sim$7–8 hours. From corrected labeling index data (text-fig. 6*b*), the rate of entry of cells into synthesis increased gradually to 40 hours, then rose rapidly, and remained between 2 and 3% per hour until 60 hours, after which time there was a gradual decline (text-fig. 6*c*). It appeared, therefore, that the rate of entry of bile duct cells into synthesis was in response to the expansion of the parenchymal cell population, and the system lagged well behind the parenchymal and littoral cell populations both in time and extent of proliferation.



Text-figure 6.—(*a*) Temporal pattern of percentage labeling indexes in proliferating bile duct cells during regeneration; mean values for 4 rats per interval; (*b*) derivation of corrected labeling indexes for bile duct cells, assuming $T_m = 1.0$ hour and $T_s = 8$ hours; (*c*) hourly rate of entry of bile duct cells into DNA synthesis determined from corrected labeling index data.

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Proliferating ductal cells were observed in clusters; some ducts appeared quite active, while others within adjacent lobules were quiescent. It may be that budding to expand the duct wall was the method of growth of the interlobular bile duct system for the increased secretory function of the growing hepatic tissue. However, during parenchymal cell proliferation, the new bile canaliculi forming within the anastomosing perforated plates of the new parenchyma were responsible primarily for the large increase in the biliary system associated with the increase in size of the liver lobules during regeneration.

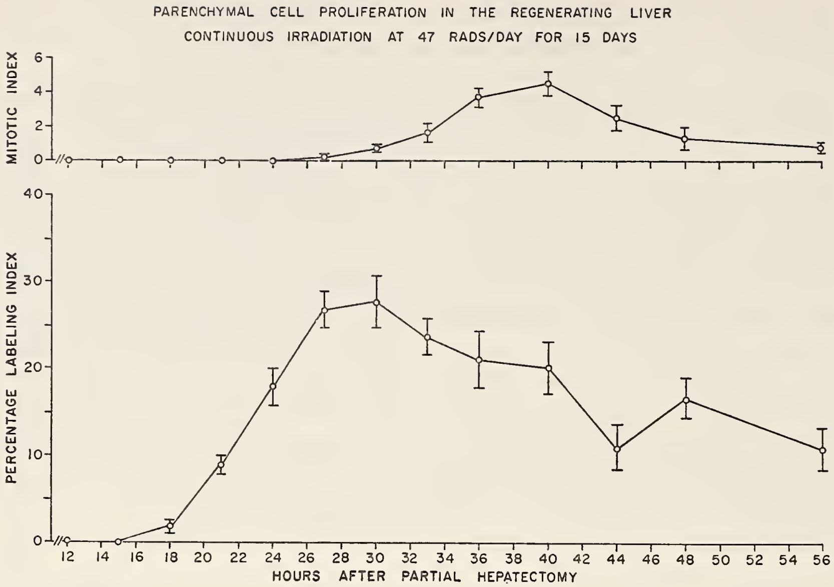
PROLIFERATION OF MISCELLANEOUS NONPARENCHYMAL CELLS

Following partial hepatectomy, connective tissue was restored very slowly; labeled periportal fibroblasts appeared at 48 hours, and their numbers were greatest between 56 and 64 hours. Mitotic activity in the fibroblasts continued for more than a week, and after restoration of the parenchymal cell population, occasional fibroblasts in division were observed. Mesothelial cells of the peritoneum were labeled at 48 hours; the labeling index reached 20–40% by 56 hours and declined abruptly thereafter. Occasionally, the vascular endothelium in the portal triads and in the large vessels was labeled between 48 and 56 hours. The onset of DNA synthesis and cell division in these different cell populations during regeneration also appeared to occur in response to the rapid expansion of the parenchymal cell population. However, growth lagged behind the parenchymal and littoral cell populations both in time and extent of proliferation, and extended over a longer period during the regenerative process.

EFFECTS OF PERTURBATION OF A CONDITIONAL CELL RENEWAL SYSTEM

The analysis of cell population kinetics in a conditional renewal system, such as the parenchymal cells in the liver, provides a valuable method for examining and comparing perturbations of the system produced by radiation or chemical agents. Our interests concern cellular response and cell population kinetics under continuous low dose-rate γ-irradiation. The following study briefly examines only one aspect of the extent to which the liver stores lethal and nonlethal cellular injury during continuous exposure, which affects the patterns of cellular proliferation and thus the speed and efficiency of regeneration (*11*, *12*). In this conditional renewal system, for example, an accumulation of latent radiation damage in the slowly proliferating cell population under continuous irradiation could become manifest as a change in parenchymal cell population kinetics during regeneration—for example, alteration in proliferation rates, the size of the proliferating population, and the duration of the phases of the cell cycle.

Text-figure 7 illustrates the pattern of mitotic and percentage labeling indexes of parenchymal cells at frequent intervals during the first 56 hours after continuous irradiation at 47 rad per day for 15 days and partial hepatectomy. Comparisons of relevant kinetic parameters are made in table 1. The delay in the onset of DNA synthesis and mitosis was approximately 5–10 hours; waves of labeled cells and mitoses were much broader than for the unirradiated animals and peaks were separated by 8–10 hours. Both curves declined more slowly than those in the unirradiated animals, and elevated levels of labeling and mitotic indexes persisted



TEXT-FIGURE 7.—Temporal pattern of parenchymal cell division and DNA synthesis in the regenerating liver after prior continuous irradiation at 47 rads per day for 15 days; mean values ± one standard error of the mean.

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to 56 hours. The fraction of cells in the liver that underwent division during this period was 20% less than normal. The duration of synthesis, determined by the method of labeled mitoses, was about 7.0 hours and the rate of entry of cells into synthesis was obtained from the percentage labeling index data, corrected for growth of the liver. Comparisons with unirradiated hepatectomized animals indicate that irradiation reduced the initial burst of cells into synthesis, the increase was more gradual, and fewer hepatocytes were involved. In both groups, maximum entry of cells into synthesis occurred within 8–10 hours after the initial appearance of labeled cells. In the irradiated animals, the subsequent steady entry of cells into synthesis decreased from the normal mean hourly rate of 4% to only 2–3%, and a gradual decline in the rate of entry occurred 8 hours later than in unirradiated animals. Allowing for the gradual decline in the rates of proliferation, the liver remnant of the unirradiated animals replaced a large fraction of the parenchymal cell deficit by 72 hours, while the irradiated animals required much more than 100 hours for equivalent restoration.

After continuous irradiation, the curve of percentage labeled mitoses was essentially the same as in unirradiated animals, although there was more scatter in the experimental points (text-fig. 2*c*). There was a slight shortening of T_s by ~1.0 hour, and a spread in the $T_{g_2+m/2}$ probably accompanied by cells held up in their passage through the G_2 phase, with a decrease in the mean duration of the phase complex by ~0.5 hour.

One mechanism that could influence the speed and efficiency of regeneration after radiation injury would be a change in the kinetics of parenchymal cell proliferation (*13*, *14*). The labeled mitoses data indicate that under continuous exposure the durations of the S and the $G_2 + M/2$ periods were reduced by approximately 1.0 and 0.5 hour, respectively. While the efficiency of cell division may have been reduced through mitotic death or the accumulation of nonlethal cell damage, the rate of proliferation apparently increased at a lower population level, thereby maintaining the regenerative capacity and influencing the speed of regeneration, *i.e.*, there was a decrease in the number of proliferating cells associated with a shortening of the durations of the $G_2 + M/2$ and S periods and there was an increase in the number of divisions during regeneration. In the unirradiated liver remnant, it appeared that most of the parenchymal cells divided only once or twice to restore a large fraction of the original cell mass within 3 days. If, after irradiation, fewer proliferating cells took part in the regenerative process, then an increase in the number of division cycles would be necessary. However, if only half of the potentially proliferative cells were capable of division, then only 3 division cycles would be necessary, and it would take less than 1 more day or so for complete regeneration to occur. Damaged parenchymal cells that have lost their capacity for indefinite division may still have been able to divide several times which would be sufficient for replacement of the cell deficit.

The speeding-up of cells passing through the S and $G_2 + M/2$ compartments represents only one mechanism for compensating for the decreased rate of entry of cells into synthesis and mitosis, even though there may have been holdup of some cells through the $G_2 + M/2$ complex period. Such a speeding-up would also compensate for the initial radiation delay in the onset of DNA synthesis and mitosis and for the decrease in the number of cells taking part in the regenerative process. The increase in the overall period of proliferation during regeneration may be an additional compensatory mechanism indicating that biochemical pathways, necessary for initiating and maintaining cell proliferation during the regenerative process, remained operative for a longer period of time after hepatectomy, thereby permitting a greater number of divisions of potentially proliferative cells at the decreased population level.

CONCLUSIONS

One of the most important biological properties common to the chemical agents and ionizing radiations used in the treatment of neoplastic disease is the capacity to interfere selectively with cellular proliferation and to damage proliferating tissues. Most of the cytotoxic agents are capable, under suitable conditions, of causing damage to growing and regenerating tissues, and leaving relatively unharmed those in which cell proliferation is not in progress. While it seems probable that neoplastic tissues are affected mainly because they are actively growing, it is evident that normal proliferating tissues are affected as well. Thus, these agents attack the bone marrow, intestinal mucosa, hair follicles, and the germinal epithelium of the skin and testis, for example, by virtue of their biological activity primarily on protein and nucleic acid synthesis and mitosis, *i.e.*, on cells in stages preparatory to division. Clearly, an understanding of cellular response and cell population kinetics in proliferating cell systems—both normal and neoplastic—is essential for the successful application of chemotherapeutic and radiotherapeutic methods in the study of cancer, *in vitro*, in the tumor-bearing animal and in malignant disease in man. Unfortunately, very few cell systems have been studied from the point of view of cell population kinetic patterns, and our knowledge of cellular control mechanisms regulating population size, proliferation, and differentiation remains severely limited. Although at present, only a few cancer chemotherapeutic agents effecting palliation and cure of clinical disease are available, newer developments in the laboratory must be anticipated as a better understanding of the kinetics of cellular proliferation is obtained, which will elucidate normal growth-controlling mechanisms and eventually the control of abnormal growth.

The conditional cell renewal system, such as the liver, provides a model of compensatory growth and regeneration; indeed, the parenchymal cell population of the regenerating liver has often been considered as a model for a synchronized or near-synchronized proliferating cell system, both with respect to DNA synthesis and mitosis. This system provides an unusual opportunity for studying the perturbations produced by chemicals and radiation, particularly with regard to effects on cellular control mechanisms and elucidation of underlying biological mechanisms. In addition, however, there are valuable practical applications of the model system for examining and comparing the action of cancer chemotherapeutic agents at the cellular level *in vivo*, for example, on changes in the patterns of kinetics of cellular proliferation and depletion. And finally, the favorable outcome of the therapeutic attack on neoplastic diseases depends largely on an increased knowledge of the proliferative and, therefore, biochemical behavior of human cancers—and it is here that radiation and drugs are valuable tools for studying biological mechanisms at the cellular level. Inevitably, empirical methods will be replaced by scientific methods as a better understanding of cellular control mechanisms concerned with the kinetics of normal and tumor cell proliferation becomes available. The result will be a greater efficiency in the application of chemical and radiation therapy for the eradication or control of neoplastic diseases in man.

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Cell Kinetic Studies of the Intestinal Epithelium: Maintenance of the Intestinal Epithelium in Normal and Irradiated Animals[1]

SAMUEL LESHER *and* JANIE BAUMAN, *Cellular and Radiation Biology Department, Allegheny General Hospital, Pittsburgh, Pennsylvania 15212*

WHEN an animal, be it man or mouse, is exposed to whole-body irradiation, one of the critical tissues upon which survival depends is the intestinal epithelium lining the inner lumen surface of the small intestine (*1*, *2*). The present paper is a discussion of the steady-state relationship between the rapidly dividing cellular component within the crypts and the differentiated functional cells covering the villi and how this relationship is affected by radiation.

Recent experiments utilizing tritiated precursors, uridine, cytidine, thymidine, and leucine, alone and in various combinations, suggest a well-defined and orderly metabolic sequence as cells move from their place of origin in the crypts to the extrusion zone at the villus tip (*3*). Cells making up the intestinal epithelium can be neatly compartmentalized (fig. 1) by their functional activity.

Within the crypt a functional compartment (F_p-zone), containing the so-called Paneth cells, lies at the bottom. The F_p-zone contains less than 10% of the total crypt population. The Paneth cells secrete a mucoprotein substance whose functional significance is not clearly defined.

Immediately above the F_p-zone is the proliferative compartment (P-zone), containing 55–60% of the total crypt population. All cells within the P-zone are in one of the 4 stages of the cell cycle at all times; about 35–40% of P-zone cells are preparing for DNA synthesis in G_1, 50–55% are synthesizing DNA in S, 7–10% are preparing for mitosis in G_2, and 5–7% are in one of the 4 mitotic phases. The mitotic process is extremely orderly, since in early prophase the cell moves out into the nuclear-free border and divides to form 2 new cells, and then the 2 newly formed nuclei move back in line along the basement membrane. This fitting of two nuclei into a space originally occupied by one nucleus forces the cells up the sides of the villus. All cell division takes place in the P-zone. When a cell moves out of the P-zone, it no longer synthesizes DNA and no longer divides.

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[1] This work was performed while the authors were members of the Biological and Medical Research Division, Argonne National Laboratory, Argonne, Ill. 60439.

An occasional mitotic figure is found in the maturation zone (M_c-zone, containing 30–50% of the total crypt population), which lies at the top of the crypt; however, this can be explained. If a cell at the top of the P-zone synthesizes DNA and is triggered into the proliferative phase of the cycle, it may be pushed into the M_c-zone before mitosis is completed. The cutoff point of the P-zone is amazingly sharp, a matter of 1 or 2 cells, above which there is no DNA synthesis and below which all cells are in some phase of the proliferative cycle. What mechanism controls the proliferative processes is unknown, although it must certainly involve an intricate balance of inhibitors and stimulators, such as Bullough's chalone complex.

When cells move from the P-zone into the M_c-zone at the top of the crypt, maturation begins; however, it is not completed in the crypt but continues as the cells move out of the crypt onto the villus (M_v-zone). Differentiation is completed in a short interval and the cells become mature and functional. Preliminary experiments utilizing tritiated uridine of high-specific activity indicate that the cells in the maturation zones (M_c and M_v) are actively synthesizing RNA. This newly synthesized RNA probably forms the enzyme templates which these cells will need when they complete differentiation and become mature and functional.

The mature, functional cells (F_v-zone) make up 75–85% of the villus epithelium. As cells move into the F_v-zone, RNA synthesis is rapidly reduced. However, the incorporation of tritiated leucine increases, which suggests that these F_v-zone cells are making enzymes needed to break down large molecules into smaller ones and to transplant these smaller molecules across cell membranes. As cells near the tip of the villus, the incorporation of RNA and protein precursors is reduced to very low levels, indicating that the cells are reaching the end of their functional lifespan.

The turnover of the intestinal epithelium is extremely rapid. If we label cells in the proliferative zone with tritiated thymidine (^{3}H-TDR) and observe their movement up the sides of the villus, we find that they reach the extrusion zone at the tip of the villus (D-zone) in approximately 48 hours in the mouse duodenum, 42 hours in the jejunum, and 36 hours in the ileum (*4–6*). The production of new cells in the intestinal epithelium is a continuous activity, exceeding that of any other *in situ* tissue component. If anything interferes with the production of cells in the crypt, the villus begins to shorten, eventually reaching a point where it can no longer hold together. Whether the inhibitory agent is a chemical, ionizing radiation, or bacteria or viruses, if the production remains blocked, the intestinal epithelium disintegrates and the animal dies.

Survival of the irradiated animal depends greatly on adequate cell production to maintain the intestinal epithelium and the blood cells which originate in the more diffused hematopoietic system. It is of prime importance to obtain complete information about the mechanisms of cell production in these 2 radiation-sensitive cellular complexes. The radiation therapist needs this information if he is to improve the therapeutic ratio, since he must kill tumor cells and at the same time produce minimum damage to the cells forming normal tissues.

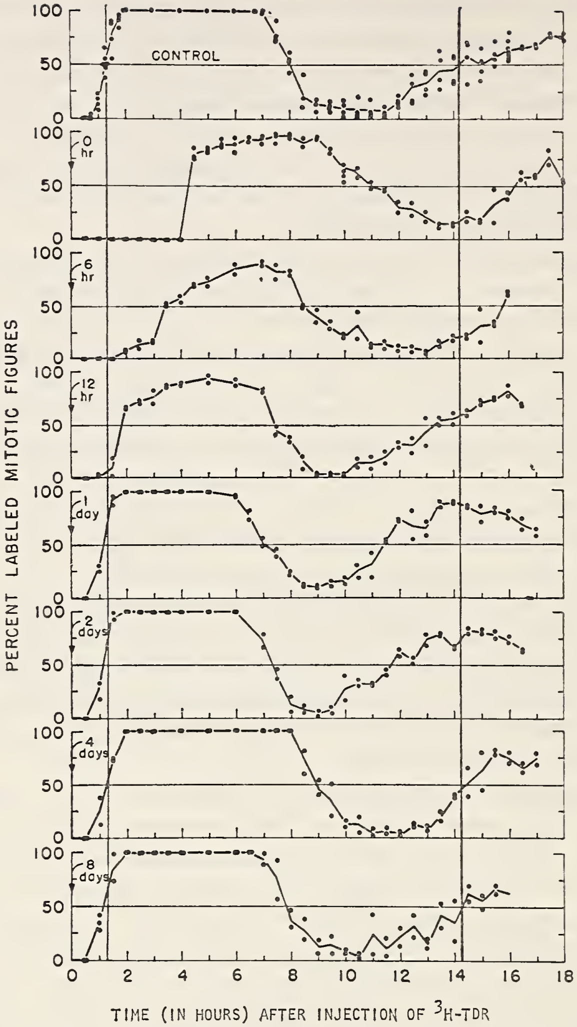
Over the past decade our research group has been concerned with irradiation effects on the intestinal epithelium of the mammalian small intestine. Most of our recent studies deal with the effects of single-dose, whole-body exposures. These studies have been concerned with the effect of irradiation on: 1) duration of the cell cycle and the four phases of the cell cycle G_1, S, G_2, and M; 2) number of cells attempting DNA synthesis (*i.e.*, the number of cells in S); 3) number of cells attempting division (*i.e.*, number of cells in M); 4) rate of DNA synthesis; 5) movement of cells from one phase of the cycle to another $G_1 \rightarrow S \rightarrow G_2 \rightarrow M$; 6) events leading to recovery of a normal steady-state relationship. From this sort of information can be drawn conclusions as to phase sensitivity.

If irradiated animals are to survive, the crypt proliferative population must be replaced, the structural integrity of the intestine must be maintained, and the steady-state relationship must be resumed. There are two ways to accomplish this, as postulated by Lajtha and Oliver (*7*): 1) by a shorter generation cycle, and/or 2) by an increase in the number of cells in the proliferative compartment.

In the first series of experiments, the effects of 300 R whole-body irradiation on the mouse duodenal epithelium were studied. From the labeled mitoses curves shown in text-figure 1, estimates of the duration of the generation cycle (GC) and the four phases of the cycle, G_1, S, G_2, and M, can be made (*8*). These curves were obtained by injecting ^{3}H-TDR into a series of mice and sacrificing them at closely spaced intervals after injection; paraffin-section slides were made and mitotic figures scored labeled or not labeled. These data were plotted against time after injection.

Table 1 shows that the duration of the generation cycles is much shorter 1 and 2 days after exposure to 300 R than that of the controls but it is back to control values by 4 days and has probably returned to a normal steady-state relationship, since it remains normal 8 days after irradiation.

The curves of 0 hour (injection of ^{3}H-TDR given immediately before irradiation), 6 hours, and 12 hours after exposure are highly distorted. As shown by the number of mitotic figures per crypt (text-fig. 2*C*) and by the 0 labeled mitosis curve (text-fig. 1), cells were blocked in G_2 for approximately 4¼ hours but, when mitosis resumed, over 70% of the mitotic figures were labeled. This suggests that cells which were in S at the time of irradiation moved into G_2 and were held there until the G_2 damage was repaired. The first plateau in all three curves never reached 100%, and the slopes of the first ascending and the first descending arms



TEXT-FIGURE 1.—Labeled mitosis curves of 100-day nonirradiated BCF$_1$ mice and 7 irradiated series of mice which received a single 300 R whole-body γ-ray dose. These curves were obtained by injecting 10 μCi of ^{3}H-TDR into mice in each series and sacrificing them at closely spaced intervals. The 0-hour group received injections of ^{3}H-TDR immediately before start of irradiation, the 6-hour group 6 hours after exposure, and the 12-hour, 1-day, 2-day, 4-day, and 8-day groups received injections at these respective times. *Each point* represents one animal.

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TABLE 1.—Estimates of the duration of the generation cycle, GC, and the four phases of the generation cycle, G_1, S, G_2, and M

Series	GC	G_1	S	G_2	M
Control	13.1	4.6	6.9	0.8	0.8
0 hour*	12.1	*0.7	6.8	*4.0	*0.6
6 hours*	12.0	*2.3	4.9	*2.8	*2.0
12 hours*	11.7	*3.5	5.7	*1.3	*1.2
1 day	10.4	2.9	6.1	0.8	0.6
2 days	11.0	3.0	6.5	0.7	0.8
4 days	13.5	4.2	8.0	0.7	0.6
8 days	13.3	5.1	6.8	0.8	0.6

*These estimates are biased, owing to the large number of damaged cells that may have difficulty preparing for division in G_2 and in completing division when they attempt mitosis (*2*).

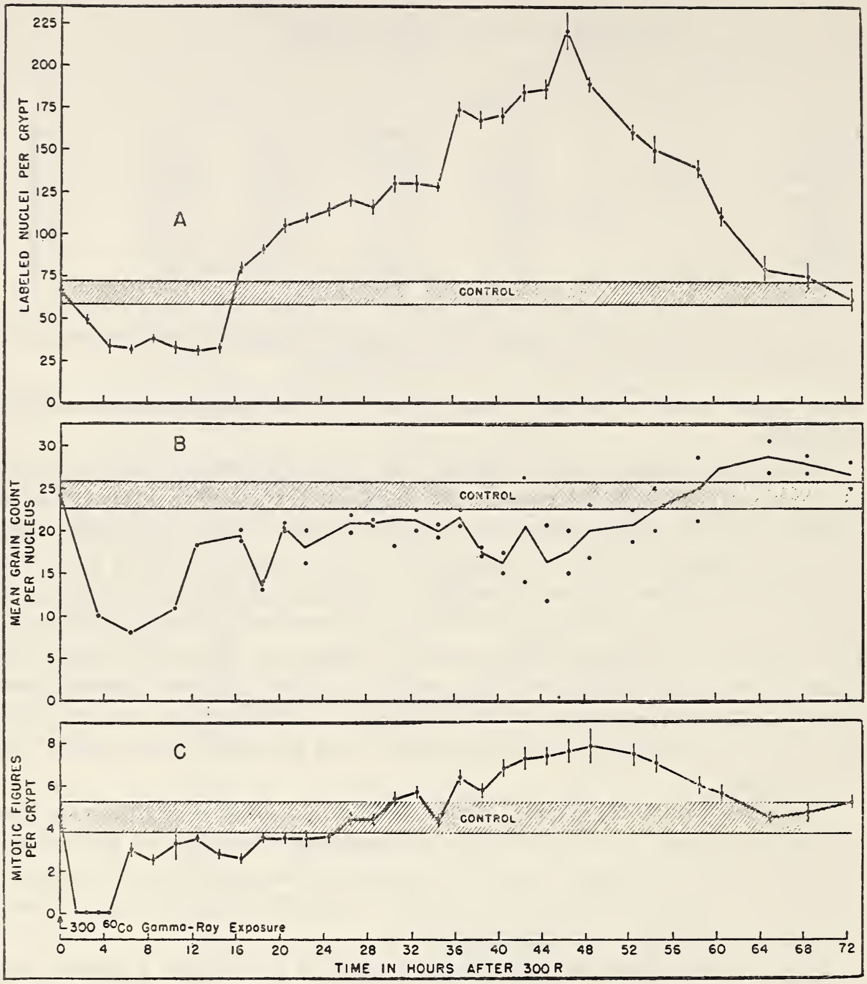
were much shallower than those found for the nonirradiated control animals.

These data suggest that, when heavily damaged cells tried to divide (particularly those in G_2 at time of irradiation), they were unable to complete the process. They then remained in mitosis for an unusually long interval, during which time unlabeled G_1 cells reached mitosis and were scored. The shallow slopes of the first ascending and first descending arms of the 0-, 6-, and 12-hour postirradiation curves are a direct reflection of the number of damaged cells and the degree of difficulty these cells experienced in dividing when they reach the mitotic phase. Mose of these cells responsible for the distorted curves are dividing for the first time and very few probably complete division but are eliminated during the first mitosis.

The labeled mitosis curve which starts 24 hours after exposure is normal in shape, although the GC is 2½–3 hours shorter than that of the normal curve. However, the cells which now make up the proliferative compartment have divided at least 2 times since irradiation; hence most severely damaged cells have been eliminated.

Examination of the labeled mitosis curves in text-figure 1 shows that, after the cells go through 2 or more divisions, the curves are not only normal in shape but also the duration of the generation cycle is reduced (1- and 2-day curves). This return to a normal production pattern at 1 day suggests that most damaged cells have been eliminated from the proliferative compartment; hence the movement of cells through the generation cycle is not noticeably inhibited. In addition to the accelerated GC, the number of cells in S increases rapidly (text-fig. 2*A*) and M (text-fig. 2*C*). The increase in LN/crypt, started at the beginning of the second postirradiation generation cycle, was from 30 LN/crypt to 80 (fig. 2). Through the next 3 generation cycles (16–36 hr), the LN/crypt reached a peak plateau, between 36 and 48 hours, of approximately 180 LN/crypts.

In a steady-state asynchronous population, the number of cells in a particular phase bears the same relationship to the proliferative population as the time spent in that phase bears to the duration of the generation



TEXT-FIGURE 2.—*A.*—Number of labeled nuclei per crypt squash (LN/crypt) in animals sacrificed at 2-hour intervals starting at 0 time, or beginning of exposure, to 48 hours post irradiation and at 4-hour intervals to 72 hours. Number of LN/crypt is an estimate of number of cells in the DNA-synthesis period S at time of injection. Two animals *per point; vertical bars* at each interval represent ± standard errors. *B.*—Mean grain count obtained by counting the grains in all labeled nuclei in each of 10 crypt squashes per animal sacrificed ½-hour after injection of 10 μCi of ^{3}H-TDR. Grain counts were made on the same preparations used to obtain the LN/crypt estimates plotted in text-figure 2*A*. These values give a relative estimate on effects of irradiation on rate of DNA synthesis. *C.*—Number of mitotic figures per crypt squash (MF/crypt) in mice sacrificed at 2-hour intervals post irradiation and at 4-hour intervals to 72 hours. Two animals *per point; vertical bars* at each interval represent ± standard errors.

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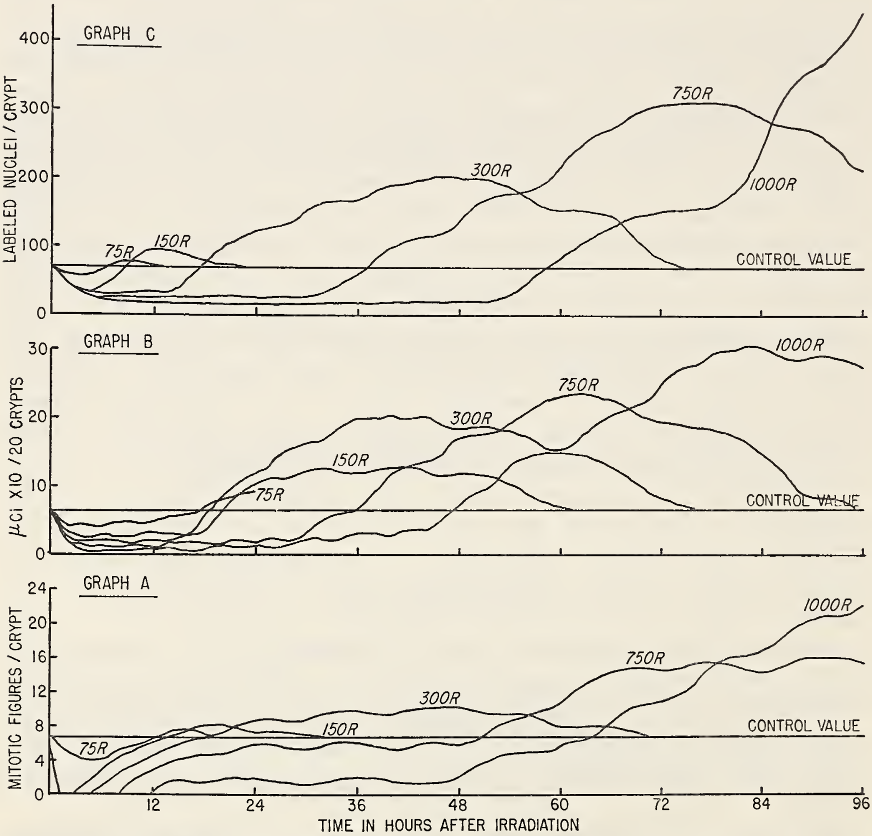
cycle. On this basis, as the number of cells in S increases from 80 at 16 hours to 115 at 24 hours and to approximately 180 at 36 hours (mean value of the 36–48-hr plateau), the total proliferative population of about 160 at 16 hours will increase to 185 at 24 hours and to 300 at 36 hours. During this 16–48-hour interval, the total crypt size changes little, which means that except for the highly differentiated Paneth cells the entire crypt belongs to the proliferative compartment during the peak production period (fig. 2).

This transitory increase in the proliferative compartment during the recovery phase does not necessarily indicate that cells that were in the upper maturation zone at the time of irradiation had regained the ability to divide. Since the number of LN/crypt increases gradually over a 30-hour period from a low of 30 to a high of 180 at 36 hours, it is reasonable to assume that during the recovery period, when radiation-damaged cells are being replaced, cells that would normally differentiate (in the maturation compartment) now synthesize another set of chromosomes and divide. Actually, one might be inclined to suggest that some of these cells may be triggered back into division, but of course this is not true. Immediately after irradiation, when cells are blocked from moving from S to G_2 to M, and also probably blocked in the early phases of G_1 (which accounts for the reduced number of cells seen in synthesis at any one time), they begin to speed up the process of proliferation and synthesizing DNA. As the cells are pushed up and away from the proliferative compartment, they move into the maturation compartment, where the control mechanism should trigger them out of the cycle. Instead of this happening, however, the cells continue to divide, and gradually, as the proliferative compartment expands, the entire crypt is involved in proliferation.

After 300 R, the production of new cells was blocked for 4½ hours and remained low for the next 10 hours. The rapid rise in the number of proliferative cells and the acceleration of the generation cycle during the next 1½ days increased cell production from an approximate low of 3 cells per crypt per hour to over 30 (normal production, 10/hr). As cells cycle through repeated divisions, those sustaining the most radiation damage are, if not repaired, eliminated from the proliferative compartment. Therefore, after each complete generation cycle, the proliferative population retains fewer damaged cells; the mouse intestinal crypt is able to re-establish a steady-state cell production pattern after 4 or 5 generation cycles.

We are now carrying on a very ambitious series of 7 different dose levels, wherein we are studying the effects of radiation at levels ranging from 75 R up to 1000 R (*9*). At 75 R, there is an immediate reduction in the number of cells in mitosis (text-fig. 3); however, since this is not reduced to 0, the G_2 block is never complete. The mitotic index (mitotic figures/crypt squash) returns to the control level very rapidly, overshoots for only a short period, and then resumes its normal value. At 150 R it reaches 0, stays there for approximately 2 hours, overshoots, and again returns to normal in about 1½ days. The effects of 300 R have already been discussed. At 750 R the mitotic index remains at 0 for about 8 hours, stays below normal for approximately 2 days, overshoots, and has not yet returned to normal as far as the experiment is carried. At 1000 R it remains at 0 from 10–12 hours, stays below normal until about 60–62 hours, and then overshoots.

As the dose increases, the degree of overshoot increases. Not only is the whole crypt involved in DNA synthesis and mitosis, but it reaches an enormous size. Crypts have been counted with over 1,000 labeled cells and well over 90 mitotic figures. Possibly 1000 R destroys some crypts entirely and only a few crypts remain to replenish the villus cells.



Text-figure 3.—Effects of various whole-body, single-dose ^{60}Co γ-ray exposures (75 R, 150 R, 300 R, 750 R, 1000 R) on DNA synthesis and mitosis in the intestinal crypts of the mouse duodenum. *A*—number of mitotic figures per crypt; *B*—rate of DNA synthesis per crypt; *C*—number of labeled nuclei per crypt.

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The number of labeled nuclei per crypt squash (text-fig. 3*C*) shows the same pattern—an immediate drop in the number of labeled nuclei per crypt, followed by a rise which overshoots the control values, then, at most dose levels, a drop back toward control levels.

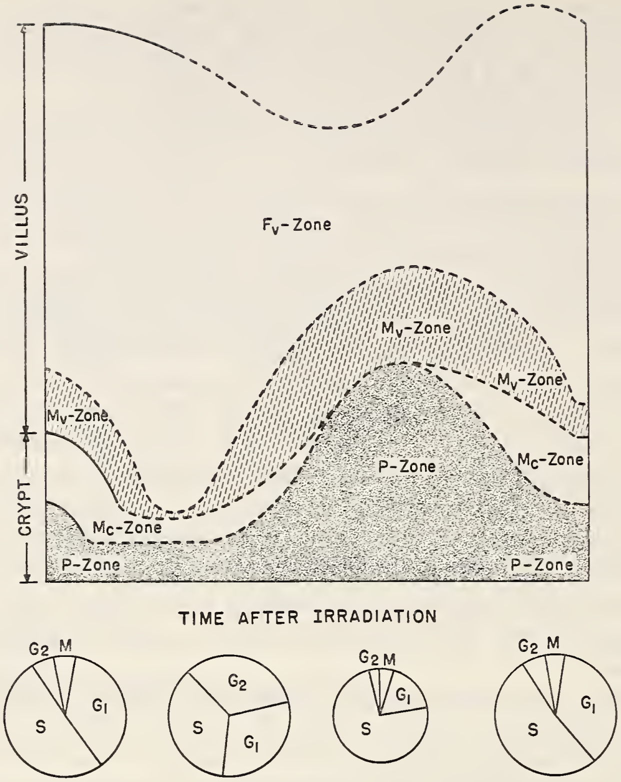
Using a micro-scintillation method developed by Dr. Walter Kisieleski at the Argonne National Laboratory to measure the incorporation of ^{3}H-TDR on a per-crypt basis as an indication of the rate of DNA synthesis, we found that the rate of DNA synthesis was affected in the same manner. The curves for each dose level are shown in text-figure 3*B*.

The preceding data are summarized in text-figure 4. Along the vertical axis is the distribution of cells in the various zones along the villus and in the crypt. Immediately after irradiation, the number of cells in the P-zone drops to approximately half at all dose levels. The maturation zone is greatly reduced in both the crypt and the villus for a time, depending on dose, and then begins a very rapid rise. At this point, the entire crypt is involved in cell proliferation, and maturation takes place only on the sides of the villus. Then, if the dose is not too high, the ratio of cells returns to the normal steady-state picture. Because the maximum lifespan of the cells on the sides of the villus is never reached in a normal animal, the change in the villus length lags behind the changes in the crypt. Even though the cells are near death, they could still serve a useful purpose for 2 or 3 more days.

The circles below the schematic model in text-figure 4 show the distributions of cells in the various phases of the cycle. The first circle shows the proportion of cells found in the four phases of the cycle in a normal animal. The second circle shows the condition immediately after irradiation when cells are blocked off from M, the number of cells in G_2 increases drastically, and the cells at the end of the G_2 block are probably almost equally divided between G_2 and S. The third circle shows a shortened generation cycle enabling more cells to be produced to repopulate the crypt and villus. The last circle indicates the cell cycle's return to its normal steady-state condition.

The reduction in the cell cycle is dose-dependent. After 1000 R at 72 hours after irradiation, the total cell cycle is less than 7 hours, with a synthesis time of around 5 hours. At this time, most cells are in S, G_2, and M. G_2 is less than one-half hour, since over 50% of the mitotic figures are labeled by the end of the first half hour. The division phase is also short, and cells spend little time in G_1 before moving into the DNA synthesis period, S.

All these changes seem to be dose-dependent. The number of cells in S drops immediately after irradiation and much more drastically following 1000 R than 300 R. The low value for S remains for a different period. After 300 R the cells in S are back to a normal number by 24 hours, whereas after 1000 R this takes 42 hours. The cells in S then begin a drastic rise and again the overshoot increases as the dose increases. At 1000 R it is 3 times what it is at 300 R, and at 300 R it is about 4 times what it is at 150 R. At low



Text-figure 4.—Generalized, whole-body effects of irradiation on the intestinal epithelium which forms the inner lining of the mouse small intestine. Immediately after irradiation there is a rapid reduction in the total number of cells in the crypt, which includes the dividing cells in the proliferative compartment (P-zone) and the differentiating or maturation compartment (M_c-zone). The number of cells remains low until most damaged cells are eliminated and replaced in the P-zone. The rate of cell production increases rapidly, and the number of cells in the P-zone exceeds control levels. When the crypt has been repopulated with viable cells, the size of the P-zone tends to return to normal. At high-exposure levels, the entire crypt cell population appears able to synthesize DNA and divide (P-zone). At the same time, the distribution of cells in G_1, S, G_2, and M is disturbed, and the generation cycle is temporarily accelerated, as shown by *lower circles*.

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dose levels (300 R and below) the cell production appears to return to a normal steady-state, but at higher dose levels (500–1000 R) cell production remains higher up to 8 days.

The increased proliferative activity by the crypt intestinal epithelium during the recovery period probably resembles that which occurs after partial hepatectomy or skin wounds. Of course, radiation not only produces lesions in the intestinal epithelium, but also it damages to some extent every cell in all organs; therefore, whole-body irradiation affects all organs. It is amazing that these crypt cells have such a tremendous potential to attempt repair and to return the steady-state population of cells to its normal cell production rate. Apparently radiation disturbs the mechanisms controlling cell proliferation. In the next series of experiments a study will be made of the effects of partial-body exposures (over the abdomen) and chemotherapeutic agents.

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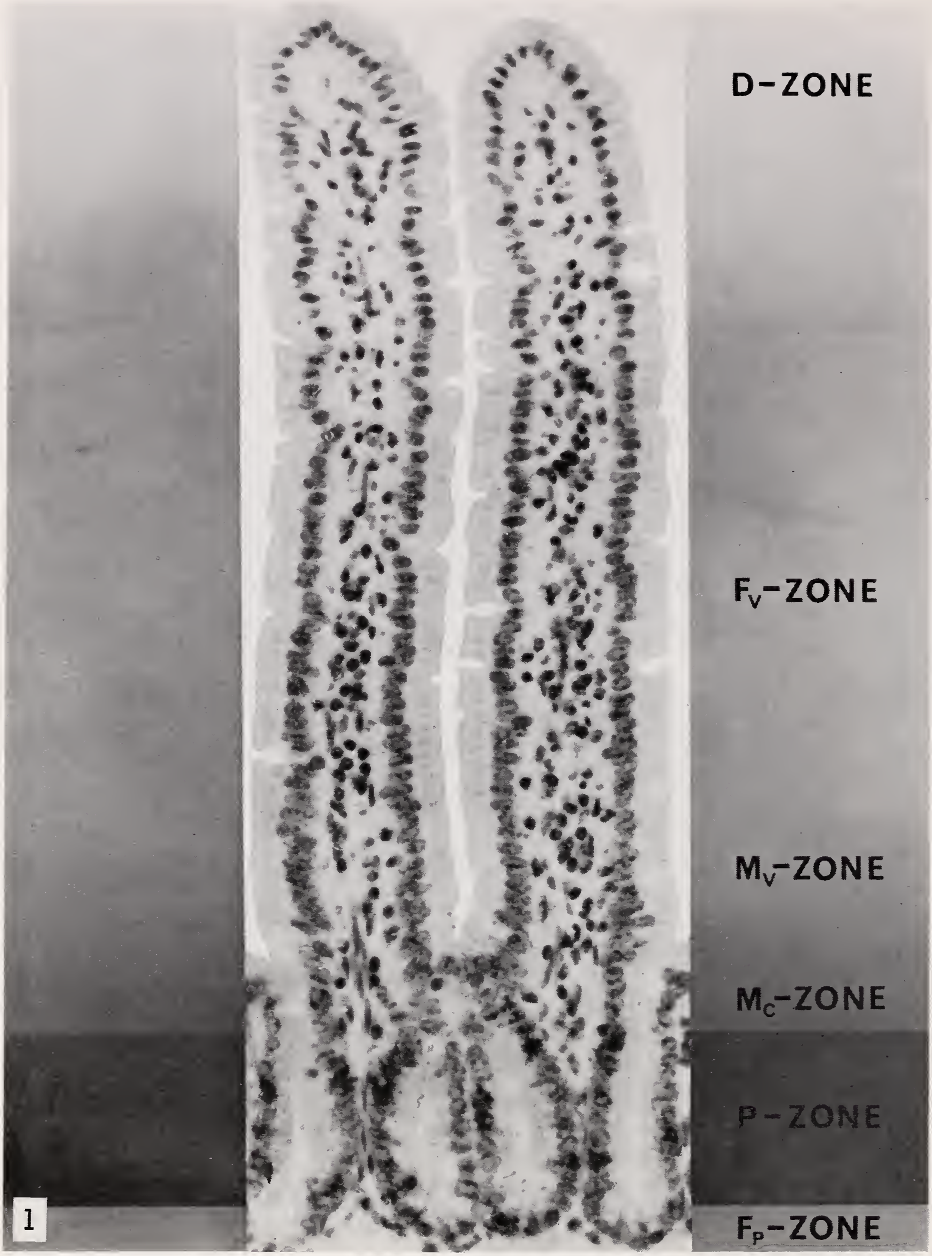

FIGURE 1.—A photomicrograph through a longitudinal cut section of the upper small intestine. The cells of the intestinal epithelium can be divided into five functional compartments: 1) F_p-zone, which contains the secretory Paneth cells; 2) P-zone, which contains the proliferative cells; 3) M_c-zone and M_v-zone, both in the crypt and villus, where all cells are undergoing differentiation; 4) F_v-zone, which contains the mature functional cells; and 5) D-zone, which contains cells near the end of their lifespan.

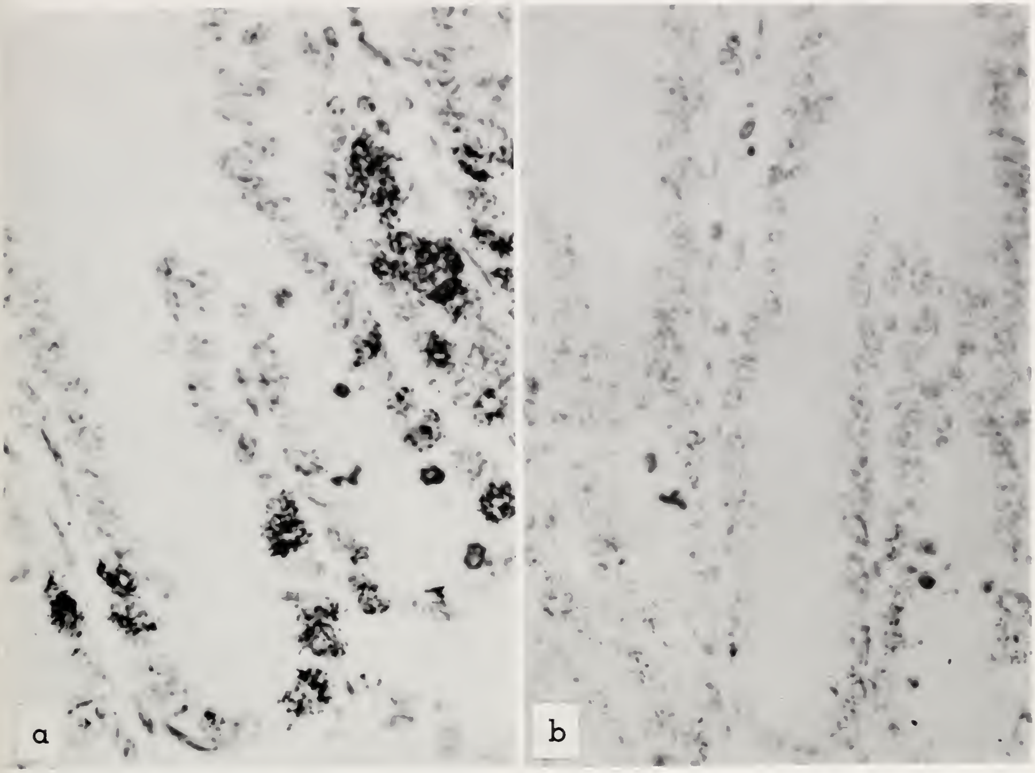

FIGURE 2.—Photomicrographs of duodenal crypt sections (3 μ). Mice were given intraperitoneal injections of 10 μCi of ^{3}H-thymidine and sacrificed 1 hour later. Autoradiographs were then prepared. Figure 2A shows a crypt section of a nonirradiated control animal; figure 2B is a crypt section from a mouse 6 hours after 300 R; figure 2C is 24 hours after 300 R; and figure 2D is 48 hours after 300 R. Comparing the degree of labeling indicates that the rate of DNA synthesis is at a very low level at 6 hours after 300 R, but increases during the peak of activity at 48 hours. The position of labeled cells shows that by 48 hours the proliferative compartment has expanded to take in the entire crypt.

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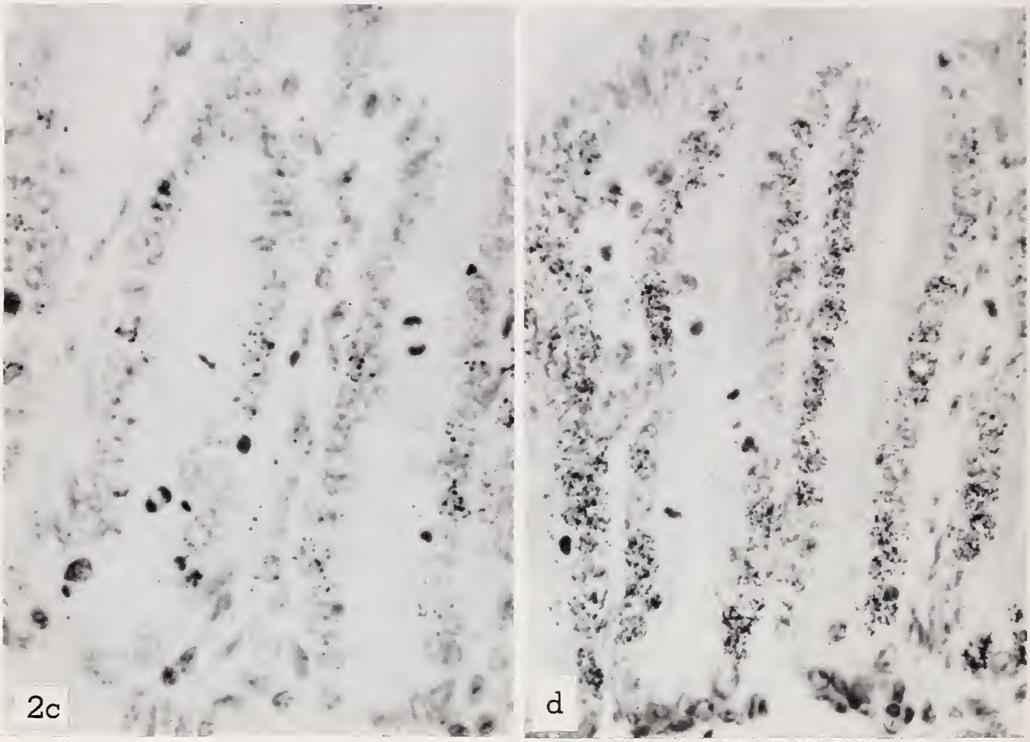
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Cell Proliferation in Gastrointestinal Disease

Martin Lipkin, *Department of Medicine, Cornell University Medical College, New York, New York 10021*

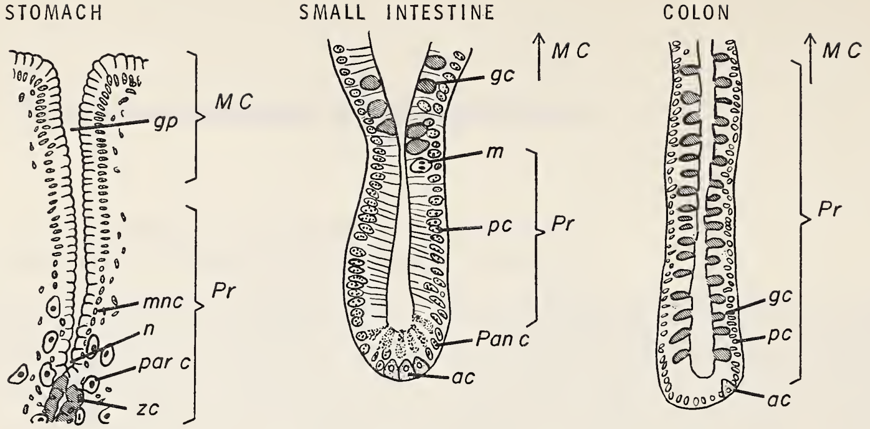
THIS PAPER describes our recent work on the proliferative behavior of gastrointestinal cells in normal and diseased mucosa. We now know that alterations in epithelial cell proliferation develop in several diseases of the gastrointestinal tract in which carcinomas are more likely to develop. These include changes in the duration of one or more phases of the proliferative cycle, the speed of replication, and the cessation of DNA synthesis in migrating and maturing cells. We are presently analyzing these characteristics to learn more about the regulatory control mechanisms governing these changes as cells progress from a normal to a malignant state.

In our studies we have used several investigative procedures. In one, tritiated thymidine (^{3}H-TDR) is injected into patients who have metastatic tumors or other preterminal diseases. Biopsies of the mucosa under study are taken at timed intervals and microautoradiographs of biopsy sections are then prepared (*1, 2*).

Another technique involves the *in vitro* labeling of epithelial cells with nucleic acid and other precursors by maintaining mucosal biopsy specimens in tissue culture media (*3, 4*). Other measurements are also made from small biopsy specimens, or from portions of mucosa removed at surgery. For example, enzymatic pathways in the intermediary metabolism of nucleic acids and the incorporation of precursors into chemically extracted DNA, RNA, and protein are being studied.

CELL TYPES IN GASTROINTESTINAL MUCOSA

In all areas of the normal gastrointestinal tract, proliferative cells are situated in the deeper portions of the mucosa (text-fig. 1). After cell division, maturing cells migrate to the surface of the mucosa and are extruded into the lumen of the gastrointestinal tract. Following cell divisions deep



TEXT-FIGURE 1.—Location of cell types in the stomach and in small and large intestine. In stomach, surface epithelium and deeper portion of gastric pit and glandular epithelium are shown. Small intestine shows crypt and lower portion of villus. Colon shows lower two-thirds of a colonic crypt. *Pr* shows area where proliferative cells and *MC* shows area where mature cells are normally found; *ac,* argentaffin cell; *gc* goblet cell; *gp,* glandular pit; *m,* principal cell in mitosis; *mnc,* mucous neck cell; *n,* neck region; *Pan c,* Paneth cell; *par c,* parietal cell; *pc,* principal cell; *zc* zymogen cell. Approximately × 250.

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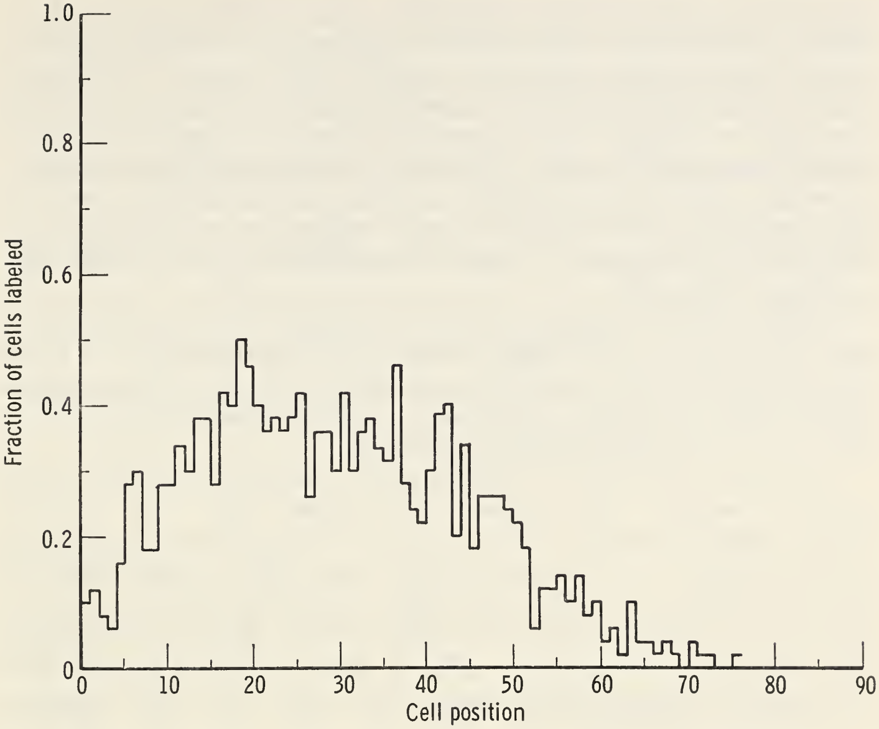
in the mucosa, two daughter cells may again divide. The frequency of cell division decreases as maturing cells migrate to the surface. At the head of the column of proliferating cells, as cells reach the anatomic area occupied by mature cells, no new daughter cells divide (text-fig. 2). In the small intestine, the proliferative cells are completely separated from the mature cells situated on the villi. In stomach and colon, proliferative cells are more directly mixed with some maturing cells; however, near the surface of the mucosa, after migration, only mature nonproliferative cells are normally found. In the esophagus, proliferative cells are also located in the basal layer (*5,6*).

After cell division, the cells enter a transitional stage during which they may or may not reenter the proliferative cell cycle. Cell reentry depends somewhat on the number of new cells needed at a given time (*7*). The sequence of biochemical events and all the regulatory signals that determine whether these cells will again proliferate, whether DNA will again be made, or whether subsequent normal maturation will take place are only partially understood.

In the crypts of the small intestine, principal, goblet, and the more slowly dividing and highly specialized Paneth cells make new DNA, then also enter into mitosis. About two-thirds of the cells in the small intestinal mucosa are nonproliferative and mature.



TEXT-FIGURE 2.—Fraction of cells synthesizing DNA (labeled with ^{3}H-TDR) 2 hours after injection in normal colonic crypts of a 50-year-old woman who had rectal carcinoma and metastatic disease. Position 1 shows the bottom of the crypt and position 90 is near and below the luminal surface. The longest crypt had 101 cell positions. Fewer cells synthesize DNA as they move above position 40 and ascend to the luminal surface.

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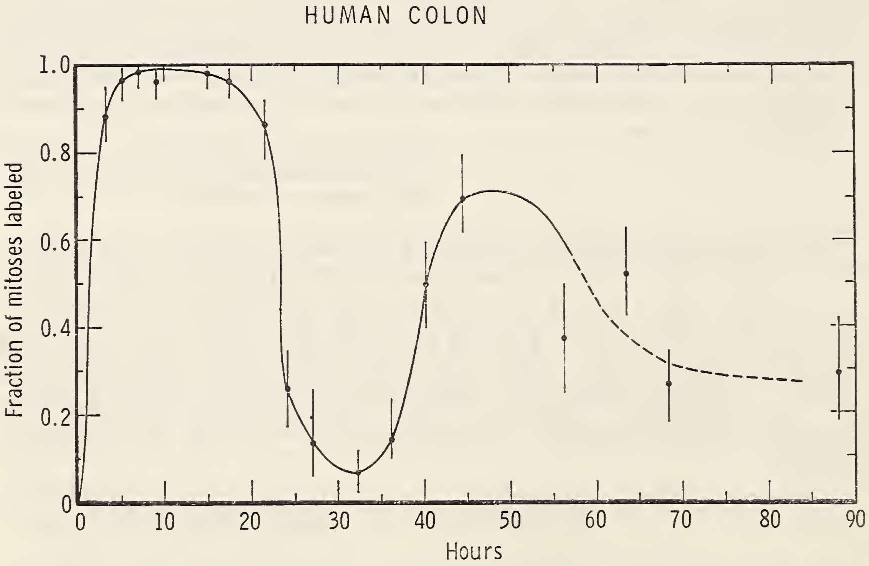
In the stomach, specialized parietal and zymogen cells are also present. In a recent study with repeated injections of ^{3}H-TDR in man, carried out in collaboration with investigators at the Brookhaven National Laboratory, we observed that parietal and zymogen cells slowly developed from proliferative cells located deep in the gastric pits. Argentaffin cells present throughout the gastrointestinal tract also proliferate at a slower rate than principal epithelial cells, and may not be derived from epithelial elements.

DURATIONS OF PROLIFERATIVE CELL CYCLE PHASES

We have estimated proliferative rates and durations of cell cycle phases after injection of either single or repeated doses of ^{3}H-TDR and the construction of labeled mitosis curves in 13 human subjects (*1*, *8–11*). An example of a labeled mitosis curve after a single injection is seen in text-figure 3. The duration of the DNA synthesis phase in proliferating gastric, small intestinal, colonic and rectal epithelial cells of man is 10–20 hours. The duration of the G_2 premitotic phase ranges from 1–7 hours, and mitosis requires about 1 hour. The G_1 premitotic duration appears to extend to about 20 hours, and the entire cell cycle is 1–2 days in proliferating gastrointestinal cells of man. Variability is evident in the proliferative rates of both individual cells and those in areas adjacent to one another in the mucosa. In the esophagus of man, the proliferative cycle is longer than mentioned above (*6*).

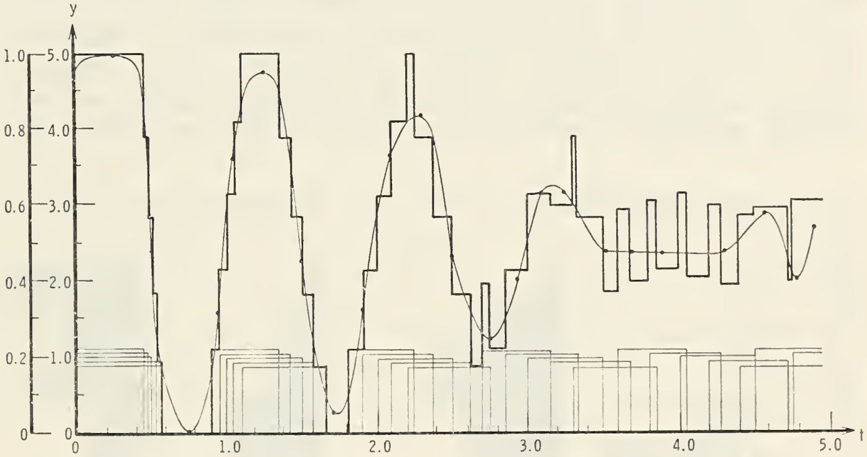
We also analyzed a labeled mitosis curve derived from a villous papilloma after injection of ^{3}H-TDR. A long G_2 duration extending to 15 hours was present. In the normal adjacent rectal issue, G_2 was normal. Studies of two rectal carcinomas revealed normal S and G_2 phase durations in one, and a very prolonged G_2 duration in the other.

Proliferative rates were in different areas of the gastrointestinal tract of newborn hamsters also comparatively analyzed (*12*). The repetitive labeled mitosis curves and rate of removal of labeled cells indicated more rapid proliferation in the small intestine than in the sigmoid colon. In the sigmoid colon, some cells reentered the prolifterative cycle after having been out of it later than cells in the small intestine. Similar findings have been observed in other species (*13*). In man, carcinomas develop with much greater frequency in the sigmoid colon than in the small intestine.



TEXT-FIGURE 3.—Fraction of mistoses labeled after injection of ^{3}H-TDR in histologically normal colonic tissue of a 50-year-old male patient. Specimens were obtained through a colostomy opening. An inoperable adenocarcinoma of the rectum was present distal to the colostomy site.

In an attempt to gain further insight into the nature of proliferating gastrointestinal cell population, we have also constructed theoretical models that simulate labeled mitosis curves and compared these to experimental data (*12*). In the theoretical models, the populations of proliferating cells are divided into subpopulations, with known amount of variation in the cell-cycle-phase durations (text-fig. 4). The contributions of the subpopulations to the total proliferating population are predetermined, as are the repetitive cycle durations of each subpopulation. These data have suggested approximately 2 : 1 spreads of cell cycle durations among proliferating gastrointestinal cell populations; they have indicated additional similarities and perturbations in the proliferative cycle of cells in the different areas of the gastrointestinal tract. As cells migrate through the proliferative zone in each area of the gastrointestinal tract, their cell cycle durations do not change markedly, and they leave the proliferative cell cycle very rapidly (*7*, *12*, *14*).



TEXT-FIGURE 4.—Repetitive labeled mitosis curves of 5 theoretical subpopulations of proliferating cells (square waves in *lower diagram*) and summated labeled mitosis curve (*upper diagram*). The square waves of the subpopulations become progressively out of phase with one another and the summated curve loses its periodic character.

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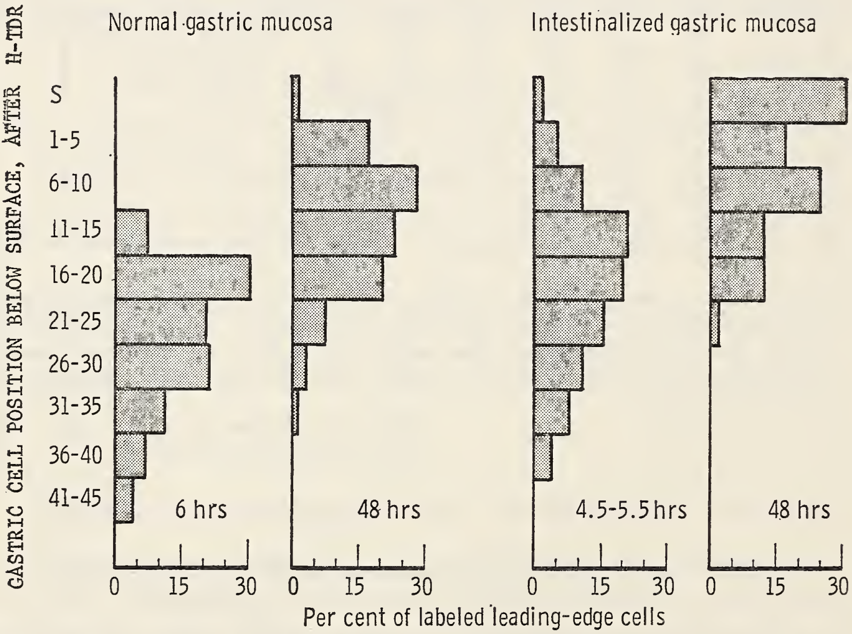
RECENT FINDINGS IN GASTROINTESTINAL DISEASE

In several diseases of the gastrointestinal mucosa in which carcinomas develop with increased frequency, alterations in proliferative activity of the cells have now been detected. In gastric atrophy with intestinalized gastric mucosa, intestinal cell populations rapidly proliferate in the stomach. In such diseased stomachs, intestinal principal, goblet, Paneth, and gastric mucous cells all incorporate ^{3}H-TDR into DNA and then enter into mitosis. In two patients, the cell-cycle-phase durations of gastric mucous and intestinal principal cells were similar to those measured in normal stomach and intestine of man. The frequencies of mitosis and ^{3}H-TDR labeling of gastric mucous cells were slightly higher than measurements made in normal stomach of man. Migration rates were also rapid (*6*, *15*).

In the mucosa of the normal stomach, proliferative cells are separated from the luminal surface by other maturing, nonproliferative epithelial cells. As the migrating cells mature and develop a higher degree of functional specialization, they cease to make DNA and no longer enter into mitosis. In other cell types in the body, proliferative activity also ceases during specialization (*5*, *16*, *17*).

In several patients with gastric atrophy and intestinalization of the stomach, examined immediately after *in vivo* injection of ^{3}H-TDR (*15*), or in *in vitro* incubation of mucosal biopsy specimens, epithelial cells labeled with ^{3}H-TDR were observed at the luminal surface of the gastric mucosa. Labeled cells were present from the neck region of the gastric glands and crypts of intestinal glands to the luminal surface (text-fig. 5 and fig. 1). The distinct zone of unlabeled maturing cells that normally separates proliferative cells from the luminal surface was absent. The



TEXT-FIGURE 5.—Cell position below surface of the gastric mucosa, occupied by the labeled mucous epithelial cell nearest the surface, 4.5–6 hours and 48 hours after injection of ^{3}H-TDR. Cell positions in normal stomach mucosa and in a patient with atrophic intestinalized gastric mucosa are shown.

labeled cells had some morphologic characteristics of well-differentiated surface cells, and epithelial cells in various stages of morphologic transition from neck to surface cells were also labeled immediately after injection. Grain counts were similar in both surface cells and proliferating cells deeper in the mucosa, suggesting that a total complement of DNA was synthesized in the surface cells.

Well-differentiated surface epithelial cells in the colon of man also synthesize DNA when hyperplasia and mucosal excrescences are present (*3*, *4*). These include multiple and familial polyposis, villous papillomas, adenomatous polyps, or hyperplasia alone. The abnormality also appears in histologically normal mucosa adjacent to mucosal excrescenses, possibly indicating the earliest sign of development of hyperplasia. In virus-induced skin papillomas of rabbits, synthesis of DNA also continues in maturing epidermal cells (*18*).

Other similarities between carcinoma cells and surface epithelial cells in some of these lesions also have been observed. Cells exfoliated from the surface of atrophic gastric mucosa have increase and variation in nuclear size, variation in size and distribution of nuclear chromatin, and resemblance to carcinoma cells on cytologic examination (*19*).

We are currently attempting to ascertain whether the continuation of DNA synthesis in surface mucosal epithelial cells in man is related to the development of carcinomas. Knowing the characteristics mentioned above, it is of interest to learn more about the cellular control mechanisms that operate during the life of the cells. Among several approaches to the overall problem, one aspect concerns the life history of the enzymes involved in the intermediary metabolism of nucleic acids needed for DNA and RNA syntheses. We are now studying enzymatic pathways that catalyze steps leading to these syntheses in normal and abnormal cells and are attempting to identify changes in the various steps as indexes of normal and abnormal maturation.

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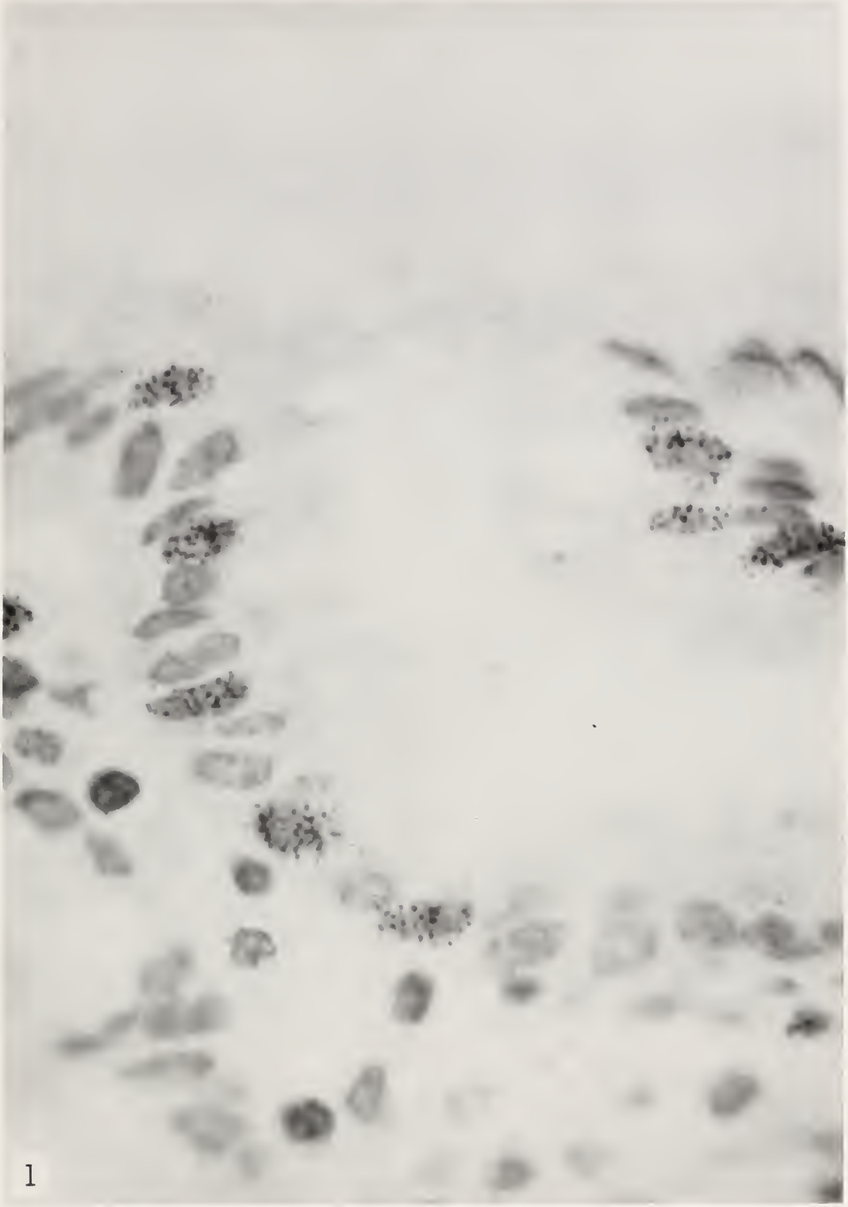

FIGURE 1.—Microautoradiograph prepared from gastric biopsy specimen obtained 1 hour after injection of ^{3}H-TDR in a patient with intestinalized gastric mucosa. Labeled gastric mucous cells are present at and near the luminal surface. × 800

In Vivo Replication of Normal and Tumor Cells: Relation to Cancer Chemotherapy[1,2]

JOSEPH POST *and* JOSEPH HOFFMAN,[3] *Department of Medicine, New York University School of Medicine and New York University Research Service, Goldwater Memorial Hospital, Welfare Island, New York, New York 10017*

THE treatment of human cancer with cytotoxins remains discouraging despite a generation of the most intensive and highly coordinated efforts ever made toward the solution of a problem in human disease. With a few exceptions, therapy has usually been marked by a high order of drug-induced morbidity, without significant amelioration of the underlying disease. Indeed, the treatment has often been associated with so much destruction of replicating normal cell populations, particularly of the gastrointestinal and hematopoietic systems, as to be attended by fatal consequences. The experiments to be described are part of a long-term study of the kinetics of normal and tumor cell replication and of the effects of cytotoxins thereupon. They have been designed to simulate the conditions in the cancer-bearing patient. Some of the data are pertinent to the clinical experiences with cytotoxins in the treatment of human cancer.

All experimental rodent tumors studied were of the carcinogen-induced, autogenous variety. They evolved relatively slowly, requiring 3–5 months. All were lethal within several weeks after achieving appreciable sizes. Three coexistent, replicating normal populations were studied in the growing rat, as a reference base for the tumor data. A few investigations were also made in cancer-bearing patients.

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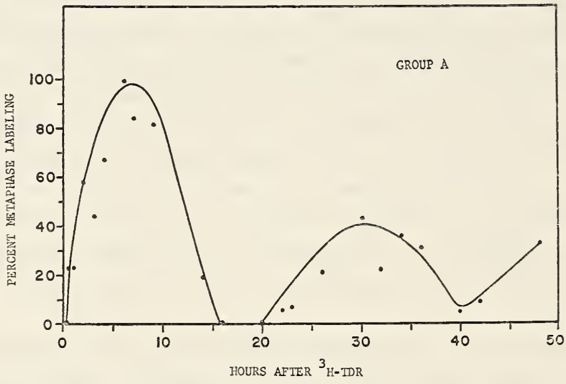
[2] Text-figures, 1, 2, 4–6, figures 1–4, and tables 1 and 2 are reproduced with the permission of the editors of the *Journal of Cell Biology, Radiation Research, Cancer Research,* and the *British Journal of Cancer.*

[3] We wish to thank Tatiana Miheyev, Judy Woo, Bruce Pachter, and Robert Sklarew for their technical assistance during the pursuit of these studies.

The method for the study of the replication of the different cell populations was first introduced by Howard and Pelc (*1*) in their work on *Vicia faba*. Later it was adapted to the *in vivo* investigation of mouse intestinal cells by Quastler and Sherman (*2*). It consists of the administration of a labeled DNA precursor, tritiated thymidine (^{3}H-TDR), and the longitudinal examination of labeled mitoses in the cell population under consideration, by the use of autoradiography. From the changing percentages of labeled mitoses, with the passage of time, the generation cycle and some of its component intervals can be determined. The size of the replicating population may be approximated from the percentage of labeled interphase nuclei. Serial grain counts over such nuclei may be used to follow labeled cohorts of cells through successive division cycles. The nuclear ploidy classes are determined microspectrophotometrically from Feulgen-stained sections (*3*, *4*).

The first population examined was that of the predominantly diploid hepatocytes in 3-week-old growing rats. The curve (text-fig. 1) showed regular polycyclical replication, with a generation time of about 21 hours and a DNA synthesis time of 9 hours. From the halving of serial grain counts, it was estimated that at least 4 cycles of uninterrupted replication occurred in this population. There may have been more, but the label became too dilute to be followed beyond 72 hours (*5*).



TEXT-FIGURE 1.—Liver of 3-week-old rat. Mitotic labeling curve (1 μc/g ^{3}H-thymidine).

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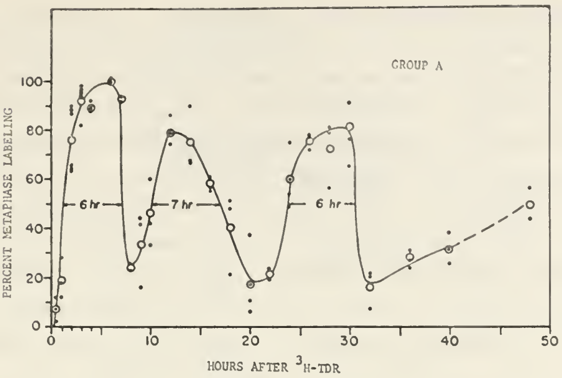
The second population was that of diploid ileal cells. The mitotic labeling curve of diploid ileal cells, coexistent with the hepatocytes, showed regular polycyclical replication (text-fig. 2). The generation time was about 9 hours and DNA synthesis required about 6 hours. Observing other ileal cells, we have found at least 4 uninterruped cycles before the label was lost (*6*). These time intervals were similar to those found by Loran and Crocker (*7*).



TEXT-FIGURE 2.—Ileum of 3-week-old-rat. Mitotic labeling curve (1 μc/g ^{3}H-thymidine). ● indicates individual rats; ○ indicates mean values.

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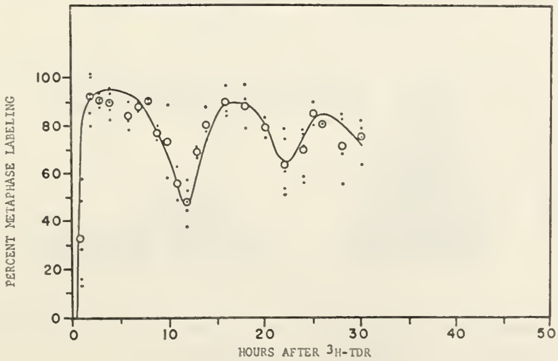
The third normal population we studied was that of diploid spleen lymphocytes (text-fig. 3) in 3-week-old rats. This curve was more complex because at least two populations of lymphocytes, large and small, were replicating and each may have had different generation times. In any case, from successive grain count halving, the initially labeled population was followed through 3 division cycles. The generation time of the first cycle was about 12 hours and DNA synthesis required about 8 hours (*8*). These findings are similar to those of Fliedner *et al.* in adult rats (*9*). The data on these 3 normal diploid populations clearly show that groups of cells replicate in regular polycyclical, rhythmic patterns, without interruption. Each cell type has its characteristic pattern and time intervals.

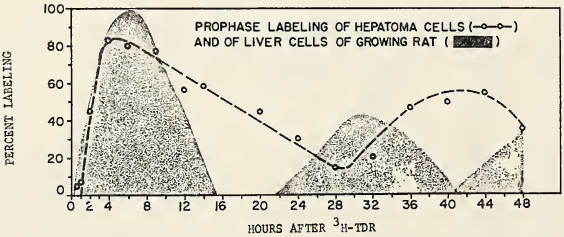
The first experimental tumor studied was the aneuploid, polyploid hepatoma, induced by the feeding of 3′-methyl-4-dimethylaminoazobenzene



TEXT-FIGURE 3.—Spleen of 3-week-old rat. Mitotic labeling curve (1 μc/g ^{3}H-thymidine). ● indicates indivdual rats; ○ indicates mean values.

(*10*). The mitotic labeling curve of these cells (text-fig. 4) was skewed and had 3 interesting features: 1) The generation time was 31 hours, 50% longer than that of normal hepatocytes, and DNA synthesis required 17 hours; 2) the second cycle followed the first, without interruption, and 3) the labeled cohort replicated as a unit. The last two characteristics are also seen in normal cells. The longer generation time was unexpected because of the widely held view that cancer cells multiply more rapidly than normal cells. The relatively orderly character of the cohort replication was also unanticipated because tumor cells are considered to grow chaotically. If such were the case, instead of the waxing and waning curve of mitotic labeling (text-fig. 4), one might expect either irregular oscillations of the curve or a rise to a plateau, followed by a leveling off until the label became too dilute to be followed. The observed data indicated that such was not the case and that tumor cells were under delicately balanced controls. The wider, skewed curve was ascribed to the greater range of DNA content of these aneuploid, polyploid cells, compared to normal diploid cells. The generation time of ileal cells, coexistent with the hepatoma cells, was 11–12 hours and DNA synthesis required 6 hours (table 1). Of the hepatoma cells, 7.5% were in DNA synthesis and 2.7% in mitosis, compared to 37.9% and 5.1%, respectively, for ileum (table 1).

The second experimental tumor studied was the rodent breast tumor. Since the hepatoma might have had unique replication characteristics, a different tumor was sought which would be relatively homogeneous and of essentially one ploidy class. The rodent breast tumor, induced by a single feeding of 20 mg of 7,12-dimethylbenz[*a*]anthracene, had these properties (*11*). The mitotic labeling curve (*12*) of these predominantly diploid cells revealed a generation time of 45 hours (text-fig. 5). The first part of the curve was relatively symmetrical, reflecting the homogeneity of DNA content of the cells. The DNA synthesis time was 10 hours. Again, a second cycle followed the first, without interruption.



TEXT-FIGURE 4.—Hepatoma. Mitotic labeling curve (50 μc/rat ^{3}H-thymidine). *Shaded background* indicates normal hepatocyte (text-fig. 1).

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TABLE 1.—Summary of data on replication and antimetabolite incorporation by normal and tumor cells

Cell type	Generation time (hr)	DNA synthesis time (hr)	G_2+ mitosis (hr)	G_1 (hr)	Ploidy class	^{3}H-TDR			^{3}H-5-IUDR	
						Mean grain count ± probable error	Interphase labeling (%)	Mitoses (%)	Mean grain count ± probable error	Interphase labeling (%)
Sarcoma (*14*)	40. 0	24. 0	1. 5	14. 5	Aneuploid polyploid	7. 2±0. 09	4. 8	0. 6	11. 1±0. 20	11. 4
Ileum (*14*)	11. 0–12. 0	7. 0	1. 0	3. 0–4. 0	Diploid	14. 8±0. 15	37. 2	6. 7	20. 2±0. 70	51. 6
Breast tumor (*12*)	45. 0	10. 0	1. 5	33. 5	Diploid	14. 6±0. 10	7. 4	0. 9	10. 9±0. 30	2. 7
Ileum (*12*)	11. 0–12. 0	6. 0	1. 0	4. 0–5. 0	Diploid	22. 2±0. 80	41. 6	6. 3	15. 5±0. 40	49. 1
Hepatoma (*10*)	31. 0	17. 0	2. 0	12. 0	Aneuploid polyploid	12. 5±0. 10	7. 5	2. 7	—	—
Ileum	11. 0–12. 0	6. 0	1. 0	4. 0–5. 0	Diploid	11. 2±0. 17	37. 9	5. 1	—	—

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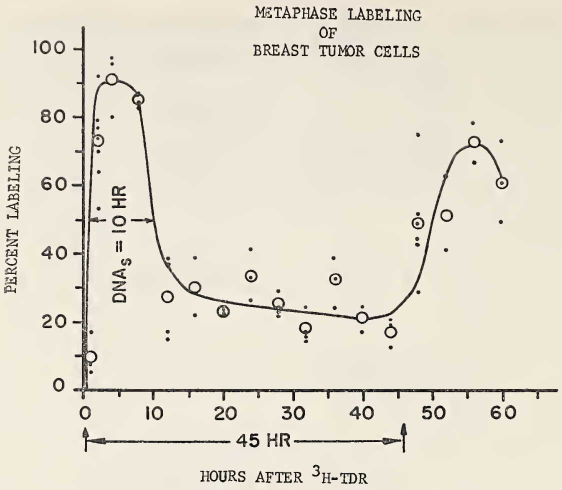

TEXT-FIGURE 5.—Breast tumor. Mitotic labeling curve (50 μc/rat ^{3}H-thymidine). ● indicates individual rats; ○ indicates mean values.

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The mitotic labeling curve for coexistent ileal cells revealed that the generation and DNA synthesis times were 11–12 hours and 6 hours, respectively.

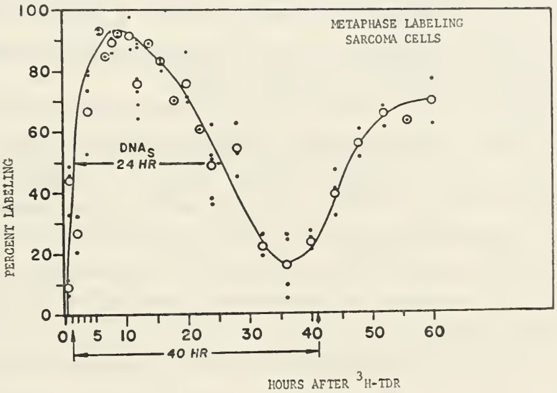
Only 7.4% of the tumor cells were synthesizing DNA and 0.9% were in mitosis. Compare these percentages in tumor cells with those in coexistent ileal cells: 41.6% ileal cells synthesizing DNA and 6.3% of them in mitosis!

The unexpected finding of long generation times for hepatoma and breast tumor cells vis-à-vis coexistent gut cells suggested a basis for the clinical experiences with the use of so many cytotoxins. If tumor cells replicated more slowly and in smaller population percentages than normal cells, then perhaps the selective cytotoxicity of antitumor agents for replicating normal cells could be related to their greater exposure to, and incorporation of, the cytotoxin.

At this point a cytotoxin, tritiated 5-iodo-2′-deoxyuridine, (^{3}H-IUDR) was administered to tumor-bearing animals to compare its incorporation by tumor and ileal cells. It was chosen for the following reasons: 1) It can be incorporated into DNA competitively with thymidine; 2) it can be readily identified in the autoradiograph; 3) from grain counts its incorporation by tumor and gut cells can be compared to that of ^{3}H-TDR; and 4) the specific cells incorporating ^{3}H-IUDR can be observed.

The incorporation of ^{3}H-IUDR by coexistent tumor and ileal cells is shown clearly in figures 1 and 2. More ileal cells incorporated the antimetabolite (49.1%) than the tumor cells (2.7%) (table 1). Also the grain counts over labeled interphase ileal nuclei were 50% higher than those over tumor cells (table 1). These data supported the hypothesis and offered a plausible explanation of how exposure to cytotoxin might operate in a model where the agent was incorporated into DNA (*12*). The larger replicating population of ileal cells, which synthesized DNA more rapidly, incorporated more antimetabolite and hence might be expected to show evidences of greater cytotoxicity (*13*).

The third tumor, studied in a similar manner, was a sarcoma of the connective tissues induced by 3-methylcholanthrene (*14*). Its cells are aneuploid and polyploid as are those of the hepatoma. The mitotic labeling curve of these cells was skewed and broad, and the second cycle followed the first, without interruption (text-fig. 6). The generation time was 40 hours and the time for DNA synthesis was 24 hours. The generation time of coexistent ileal cells was about 12 hours and the DNA synthesis time was 7 hours. Only 4.8% of the sarcoma cells were synthesizing DNA and 0.6% were in mitosis, compared to 37.2% of the ileal cells synthesizing DNA and 6.7% in mitosis (table 1).



TEXT-FIGURE 6.—Sarcoma. Mitotic labeling curve (50 μc/rat ^{3}H-thymidine). ● indicates individual rats; ○ indicates mean values.

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The incorporation of ^{3}H-IUDR by coexistent sarcoma and ileal cells is shown in figures 3 and 4. Only 11.4% of sarcoma cells were labeled, compared with 51.6% of ileal cells. The grain counts over the nuclei of ileal cells were twice as high as those over the tumor cells (table 1).

Clearly, all three tumors had cell populations with relatively long generation and DNA synthesis times when compared to their coexistent ileal cells. Also, 2 hours after the administration of the labeled precursor, the levels of grain counts in the several populations were consistent with the respective DNA synthesis times. The diploid ileal cells had higher grain counts than either diploid breast tumor or polyploid sarcoma cells. Even though polyploid hepatoma and coexistent diploid ileal cells had similar grain counts suggestive of comparable DNA synthesis rates, hepatoma cells with more DNA to synthesize would have a longer DNA synthesis time. This agrees with the observed synthesis times. In addition, much larger percentages of the normal ileal cells were replicating than the respective coexistent tumor cells. Higher mitotic indexes were always found among ileal cells (table 1). It is noteworthy that the ileal cells in all 3 groups of tumor-bearing animals had similar replication kinetics. However, the tumor cells had considerably different generation times. Finally, tumor cells had a well-structured and orderly pattern of cohort replication as had normal cells. Lower percentages of tumor cells incorporated the antimetabolite and in reduced amounts when compared to coexistent normal ileal cells.

A major void in tumor biology is the paucity of data on the replication kinetics of human cancer cells. There are a few studies, such as those of Killmann *et al.* (*15*), in which serial bone marrow samples were obtained from patients with myeloma, after the administration of ^{3}HTDR. From grain count halving the generation times were calculated at 48–144 hours. Similar results were obtained with leukemic cells (*16*). Clarkson *et al.* (*17*) reported on mitotic labeling curves of tumor cells metastatic to ascitic fluid. They found relatively long cycle times.

Our own studies in this area are much more modest (*18*). At the time of surgery, elderly tumor-bearing subjects with advanced malignancy were given very small labeling doses of ^{3}HTDR. The resected tumor and adjacent normal tissues, as well as lymph nodes, were often available for examination. Autoradiographs were made, and labeling indexes, mitotic indexes, and grain counts were estimated in normal and tumor cells. In addition, the ploidy classes of the several populations were determined. The data obtained from normal and tumor cells were considered only as relative to each other. This method was used, since multiple samplings of solid tumors could not be obtained. Seven patients were studied (table 2). The mitotic indexes of the normal tissues of origin were the same or slightly higher than those of tumor tissues; they were never lower. The percentages of labeled normal cells were sometimes higher than those of the tumor cells, but never lower. In 5 of 7 tumors, the grain counts were significantly lower in the tumor cells than those in the normal tissues of origin and of the lymph node lymphocytes. Inasmuch as many tumors were polyploid, these differences are of even greater significance than one might judge from the grain counts. Thus, if a polyploid tumor cell contains 2 or 3 times the amount of DNA in a normal diploid lymphocyte or diploid intestinal cell, and if both cell types have the same grain count 2 or 3 hours after ^{3}HTDR, clearly the time required for DNA synthesis in the tumor cell would be considerably longer than that for the normal cell. Interestingly, the diploid intestinal cells and diploid lymphocytes had similar grain counts (table 2).

From these limited data we have concluded that larger percentages of human normal cell populations are often replicating than are coexistent

TABLE 2.—Grain counts, interphase labeling, and mitotic indexes in normal and tumor cells (*18*)

	Case	Time after ^{3}H-TDR	Mean grain count ± probable error of mean	Labeled interphase (%)	Mitosis (%)	Ploidy class
64–20	Normal rectum	2.75	14.7 ± 0.22	32.1	4.0	Diploid
	Carcinoma	2.75	8.2 ± 0.15	21.7	2.9	Diploid
64–13	Normal colon	2.0	12.9 ± 0.12	32.1	4.7	Diploid
	Lymphocytes in normal node.	2.0	11.6 ± 0.13	—	—	Diploid
	Carcinoma	2.0	14.5 ± 0.26	14.5	2.9	Diploid
64–30	Normal colon	2.0	14.7 ± 0.17	13.9	1.9	Diploid
	Lymphocytes in lymph nodes.	2.0	14.1 ± 0.13			
	Carcinoma	2.0	8.3 ± 0.18	13.0	2.1	
	Metastatic tumor cells in lymph node.	2.0	8.8 ± 0.25	—	—	—
64–22	Normal stomach	3.75	14.4 ± 0.09	15.4	3.2	Diploid
	Carcinoma	3.75	10.8 ± 0.14	9.9	2.3	Aneuploid, polyploid
64–27	Lymphocytes in normal node.	6.5	14.0 ± 0.24	—	—	Diploid
	Carcinoma, oral pharynx.	6.5	11.4 ± 0.23	8.3	1.7	Aneuploid, polyploid
64–21	Lymphocytes in lymph node.	2.5	14.2 ± 0.29	—	—	Diploid
	Carcinoma, parotid.	2.5	7.1 ± 0.29	3.8	1.6	Diploid
64–29	Carcinoma, ovary	0.75	7.9 ± 0.13	2.2	0.7	Aneuploid, polyploid

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tumor cells and that the DNA synthesis time of the normal cells is shorter than that of tumor cells. Again, however, these data are all relative. While no estimates of generation times may be made, it would seem reasonable to conclude that tumor cells with longer DNA synthesis times would have longer generation times. These data are consistent with those obtained from the 3 animal tumors.

If one accepts these conclusions, clearly tumor cells growing in a setting of more rapidly replicating normal cells, such as in a lymph node or in the bowel, do not derive their growth advantages from their more rapid multiplication. This is not always so, for in the rodent hepatoma or metastatic tumor to adult liver, the tumor cells, however slowly they replicate and in however small a cohort size, are dividing in a background of adult liver cells which have an even lower replication rate (*19*).

The data presented justify several conclusions. Tumor cells may be considered among the class of cells that are continuous replicators, such as those of gut or of the hematopoietic system, since they usually divide throughout the life of the host. Tumor cells share several properties of such normal cells. 1) They replicate as wave-synchronized cohorts, with one cycle following the previous one, without interruption. 2) While the several tumor cell populations differ with regard to the time components of their respective cycles, there is sufficient uniformity within a particular tumor cell population for it to have its own characteristic mitotic labeling curve. Normal cell populations behave similarly. These data indicate that there are precisely regulated controls exercised on the metabolic events involved in the replication of tumor as well as of normal cells. 3) Tumor cells replicate more slowly than coexistent normal cells. 4) Smaller population percentages of tumor cells than of coexistent normal cells are usually engaged in replication. These findings have suggested a basis for understanding the shortcomings of cancer treatment with cytotoxins, inasmuch as the use of cytotoxins has been based on the assumption that tumor cells replicate more rapidly than normal cells. In our experimental model, it is clear that, at a particular level of circulating antimetabolite, perhaps 8 or more times the population percentages of intestinal cells than of tumor cells are exposed to the antimetabolite during a given time. Further, during one cycle of tumor cell replication, 3 or 4 cycles of ileal cells will have taken place. Finally, more antimetabolite is incorporated by the normal cells, with shorter DNA synthesis times, than by tumor cells. These data suggest that the rate of the metabolic activity, in which the antimetabolite is involved, defines the limits of the competitive incorporation of the cytotoxin. In the case of IUDR, it is DNA synthesis. In turn, the amount incorporated (*20*, *21*) and the size of the cellular population so involved would determine the net toxicity of the compound. Thus, the renewal patterns of autogenous tumor cells in relation to coexistent replicating normal cells could account for the limits of usefulness of this form of therapy.

On the other hand, transplanted tumors, which have been used widely as the test model for the study of the effects of antimetabolites, grow relatively quickly and their cells have replication rates similar to those of coexistent normal cells [*see* table III in (*12*)]. Thus, Ehrlich ascites tumor cells transplanted into the mouse may have the same generation time as murine duodenal cells, namely about 18 hours. In such a setting, the tumor cells might compete successfully with normal cells for the incorporation of, or exposure to, the cytotoxin and the cytotoxin might appear effective against the transplanted tumor cell population. However, when used in patients with slowly replicating autogenous tumor cells, the antimetabolite might prove too toxic for the more rapidly multiplying normal cells to be tolerated as an antitumor agent. This has been the history of many cytotoxins.

The proposed relationship between cell replication kinetics and cytotoxicity, as the basis for the action of antitumor agents, may be an oversimplification of the problem. However, it is useful in explaining the common clinical experiences with antimetabolites, in which replicating normal cells are invariably destroyed whether or not tumor cells are affected. This construction suggests that if cytotoxins are to become effective antitumor agents in the way that antibiotics have become useful antibacterial agents, specific metabolic attributes of particular tumor cells must be found which can be exploited.

The limited data available clearly show that all types of tumor cells may not be considered as a single biological entity any more than all varieties of normal cells may be so classified. Indeed the therapeutic benefits from the use of certain antimetabolites in some patients with lymphoma and leukemia suggest either that the tumor cells have unique metabolic properties which render them selectively sensitive, or that the generation times of these cells are similar to those of replicating normal cells. This last relationship would simulate that previously described for transplanted animal tumors.

If the dynamic behavior of human tumors is to be understood, more information is needed than that offered by the traditional examination of the stained histological section. Basic problems in tumor cell biology, such as the generation time and its component time intervals, the size of the pool of replicating tumor cells, the number of replications per cohort, the death rate of tumor cells, and, perhaps, the changing replication rates during the development of autogenous tumors, all require close examination. Such studies should lead to new and more fruitful approaches to tumor control.

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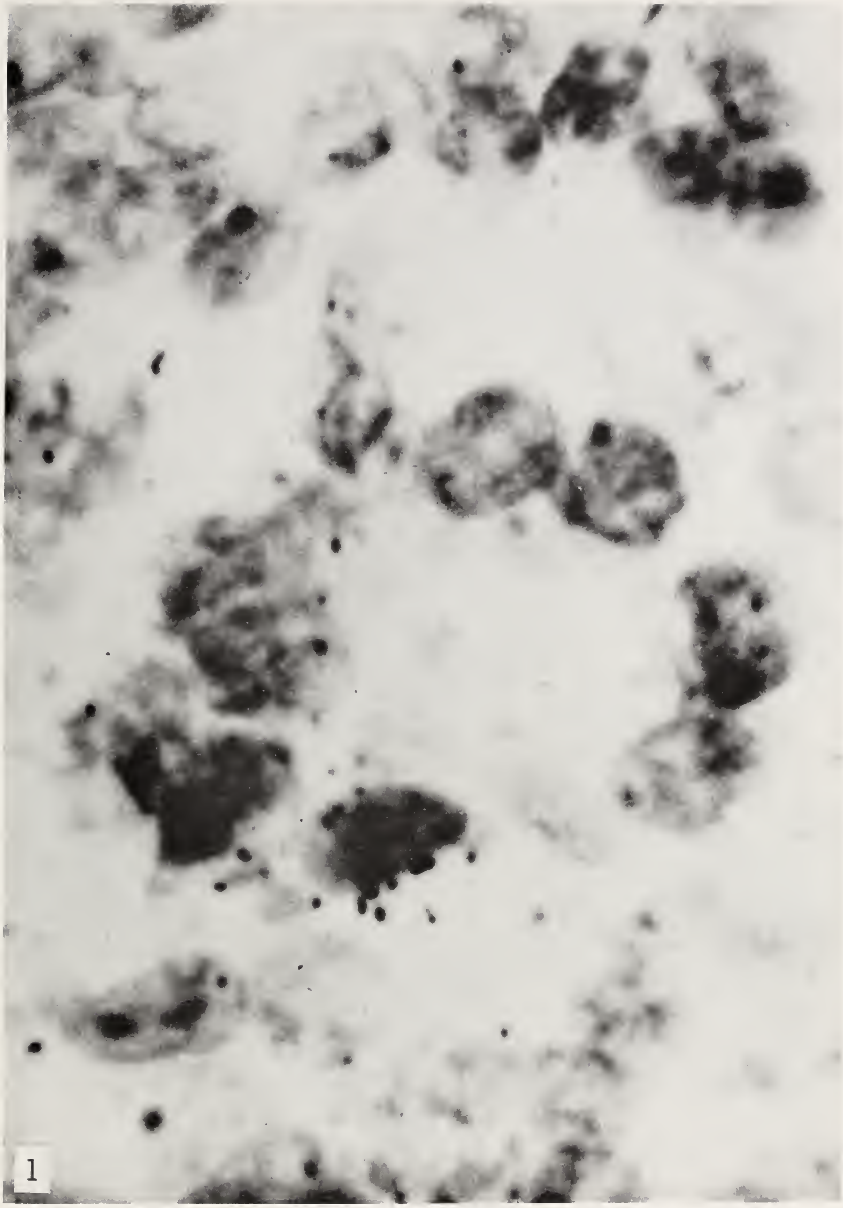

FIGURE 1.—Breast tumor. Cells labeled with tritiated 5-iodo-2′-deoxyuridine.

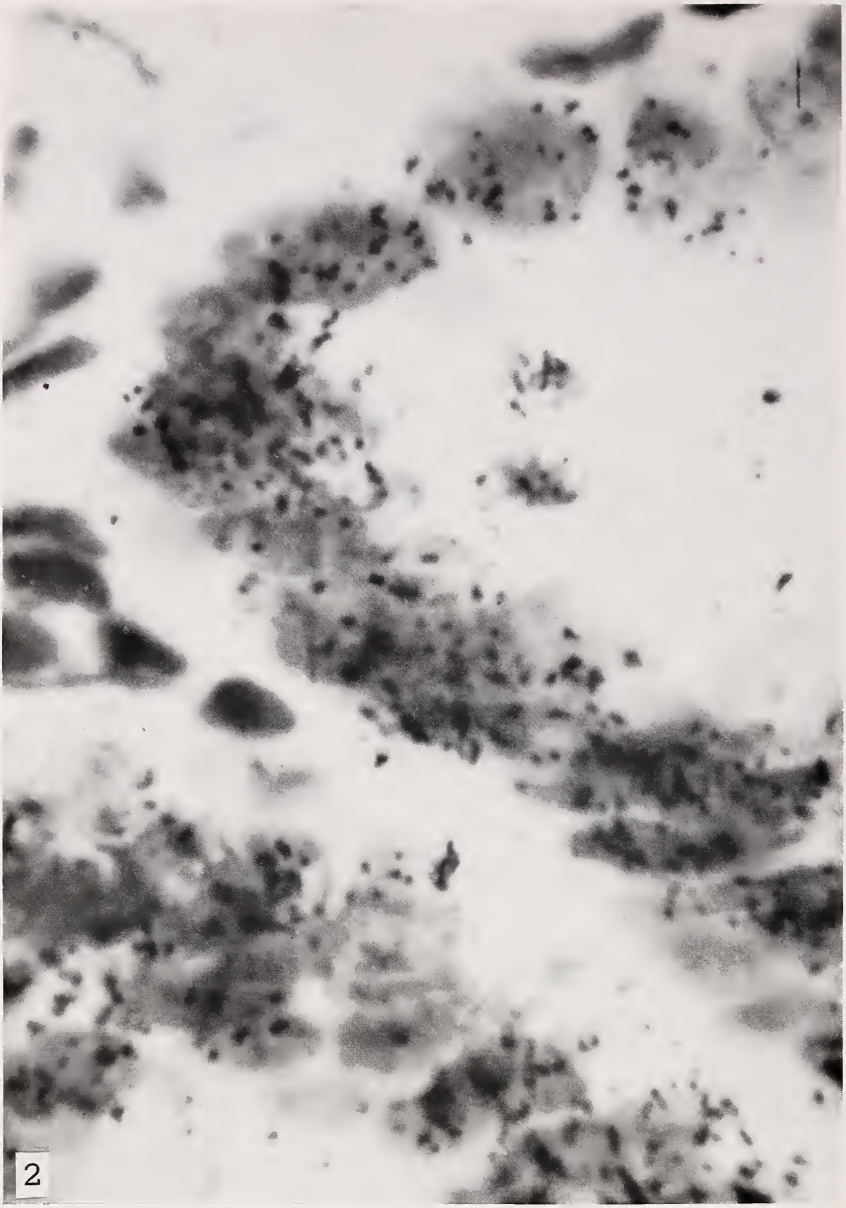

FIGURE 2.—Breast tumor. Ileum. Crypt cells labeled with tritiated 5-iodo-2′-deoxyuridine.

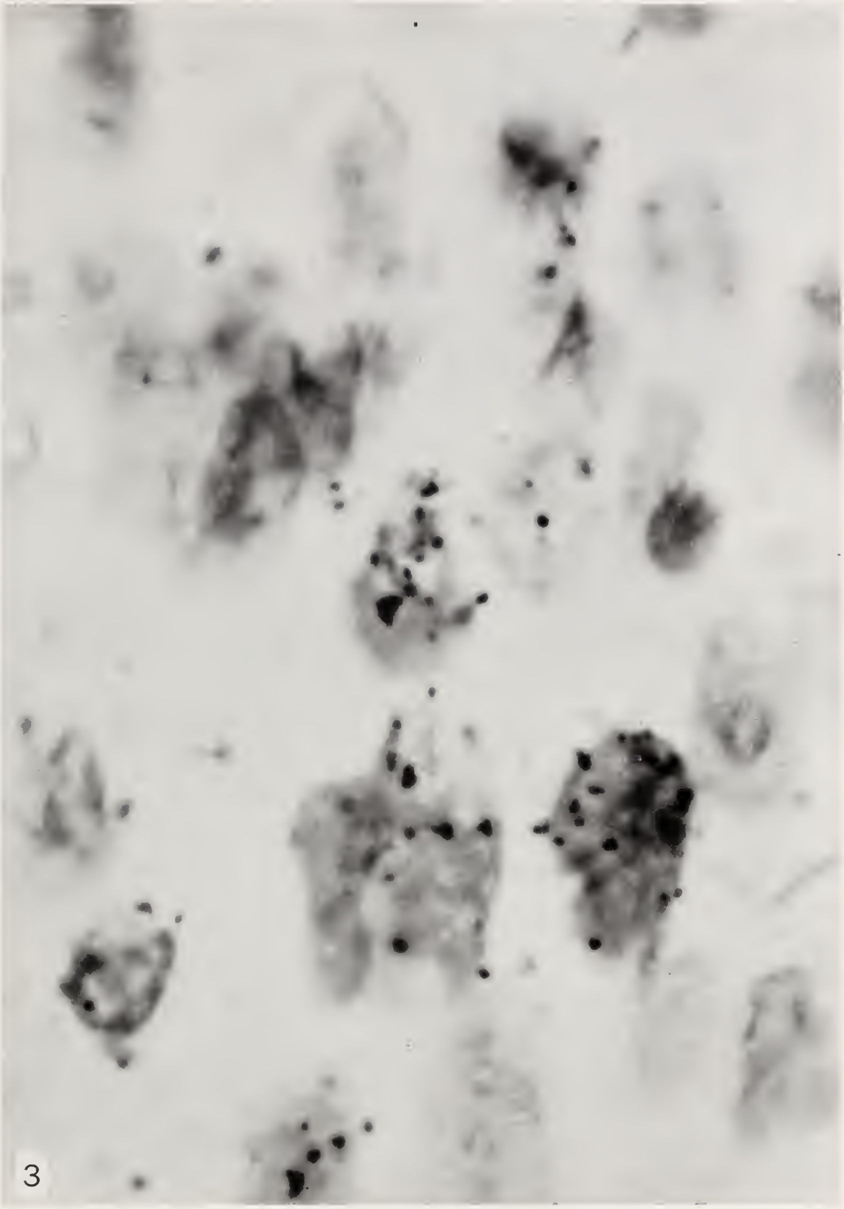

FIGURE 3.—Sarcoma. Cells labeled with tritiated 5-iodo-2′-deoxyuridine.

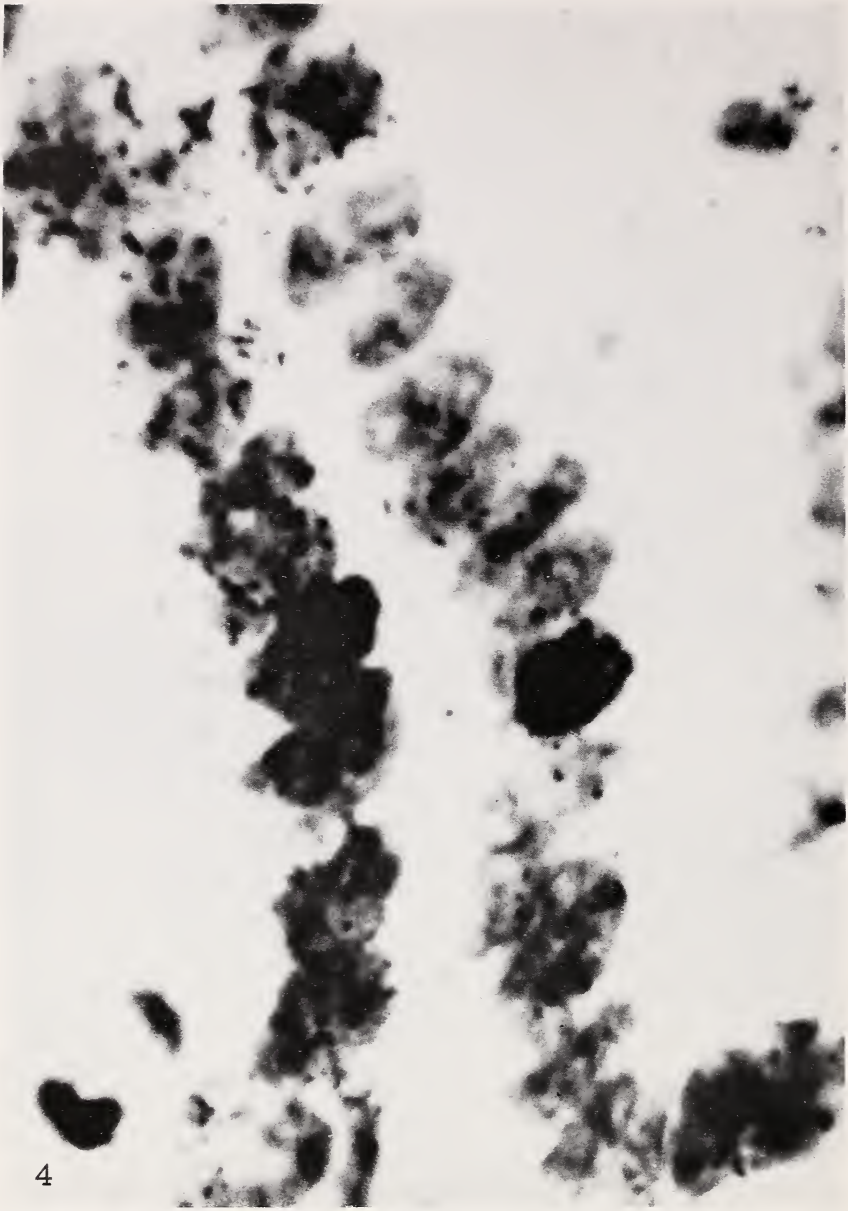

FIGURE 4.—Sarcoma. Ileum. Crypt cells labeled with tritiated 5-iodo-2′-deoxyuridine.

Cell Proliferation Kinetics in Benign and Malignant Skin Diseases in Humans[1]

Gerald D. Weinstein, *M.D.*, *and* Phillip Frost, *M.D.*, *Department of Dermatology*, *University of Miami School of Medicine*, *Miami*, *Florida 33136*

OUR INTEREST in cell proliferation kinetics developed while studying patients with psoriasis at the Dermatology Branch, National Cancer Institute, several years ago. Since then, we have extended our studies of cell proliferation to other conditions of the skin, such as the ichthyosiform dermatoses, neoplasms (both primary in the skin and metastatic to the skin), and hormonal effects on skin. Much of this work is still in its early stages, but will, hopefully, provide some meaningful data on which to plan future studies.

Psoriasis, a benign skin disease, is manifest clinically as patches of thick, white scales on erythematous skin. Psoriasis may involve varying quantities of body surface and can be quite discomforting. It is dominantly inherited, with incomplete penetrance. Patients with extensive psoriasis can now be treated quite successfully and dramatically with the folic acid antagonist, methotrexate (*1*). Figure 1 shows a patient with over 80% body involvement prior to receiving methotrexate. After 8 weekly intramuscular injections of 50 mg, the patient is almost completely clear of lesions (fig. 2). The use of methotrexate, unfortunately, is not a cure but a controlling type of therapy. The patient requires repeated injections of methotrexate to maintain this improvement. Such therapeutic responses stimulated our interest in how methotrexate affects this disease.

The clinical observations that excessive amounts of scale were produced rapidly and that psoriasis responded to chemotherapeutic agents suggested a pathophysiologic process of increased tissue reproduction. Rothberg *et al.* (*2*) and Van Scott and Ekel (*3*) were first to measure an increased turnover rate in psoriatic epidermis as compared to that of normal skin. With the techniques which utilize local injections of tritiated thymidine, we are able to study the turnover of these tissues at the cellular level in greater detail.

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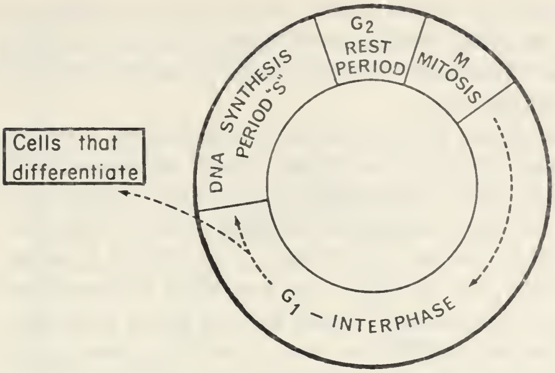
The histology of normal and psoriatic skin is compared in figure 3. The dermis, composed primarily of collagen, is relatively inactive with regard to cell proliferation. Our studies are concerned with cell proliferation in the epidermis. The epidermis can be divided into three cellular compartments: 1) the basal cell layer, the bottom row of the epidermis containing the germinative cells; 2) the stratum spinosum, the differentiated viable cell compartment above the basal layer; and 3) the stratum corneum, the superficial exfoliating cell compartment. In contrast to the histology of normal epidermis, that of psoriasis reveals a much thicker (acanthotic) epidermis containing many more cells distributed in a germinative compartment several rows deep, a larger differentiated cell population, and a much thicker stratum corneum.

It is the stratum germinativum, or basal layer(s), which is assumed to be the proliferative compartment of the epidermis. On the average, from each dividing basal cell one of the two daughter cells will remain in the basal layer and the other will move upward. Experimental evidence has been presented by Leblond *et al.* (*4*) accounting for the epidermis being maintained at the same thickness. Experimental data will be presented to show that the movement of cells through the stratum spinosum compartment normally requires about 12–14 days. Various experiments for determining stratum-corneum turnover time will be discussed. Turnover times of the latter two compartments are dependent on their size, but mainly reflect the rate of cell proliferation and mitotic behavior in the germinative compartment, which acts as the "motor" for the epidermis. Techniques and data to measure proliferation rates of basal cells will be described.

A general scheme of the cell cycle appears in text-figure 1. For these experiments, patients are exposed *in vivo* to tritiated thymidine, which is incorporated into cells that are in the process of DNA synthesis. Five μc of tritiated thymidine is injected intradermally into multiple sites of skin or skin lesions (*5*). Each one of these sites is biopsied at serial intervals thereafter. Specimens are prepared in the usual histologic fashion and exposed for autoradiography. The advantage of using tritiated thymidine by this local technique is that only a very small amount of isotope is needed. Five to 100 μc can be used per patient in contrast to 5–20 mc needed with intravenous injections.

Figure 4, an autoradiograph of the normal epidermis, shows 3 labeled cells in the basal layer 1 hour after the injection of tritiated thymidine. In sequential biopses every few days, upward migration of labeled cells is observed until an endpoint, the top of the viable cell population (the granular layer), is reached. At 6 days after injection, labeled cells have moved up approximately midway through the epidermis. At 12–14 days, the first labeled cells have reached the top of the viable epidermis (fig. 5).



TEXT-FIGURE 1.—The cell cycle is divided into four phases, the length of each being constant for a specified population of cells. Following mitosis, a certain time is spent in interphase (G_1), during which the cell is involved in various metabolic activities. It then enters the DNA synthesis period (S), during which it doubles its complement of DNA in preparation for mitosis. A short post-DNA synthesis period (G_2) prior to mitosis follows. Of the two daughter cells from mitoses, on the average, one will remain in the germinative cycle to divide again, while the other will differentiate and move outward through the epidermis.

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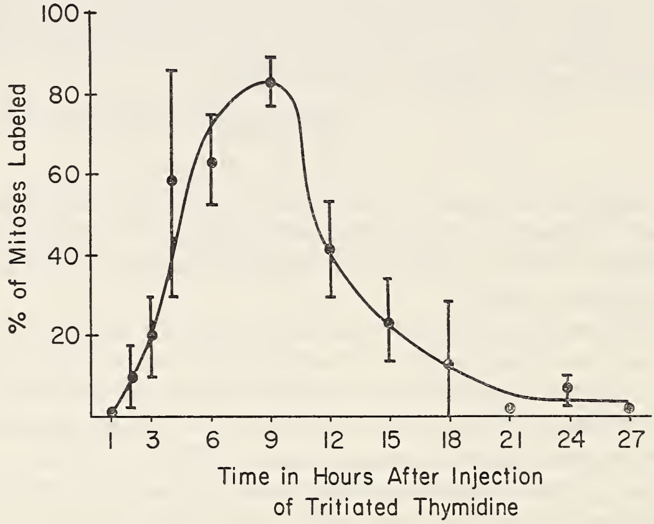
This is considered to be the transit time through the differentiated cell compartment. Unfortunately, labeled cells cannot be followed through normal stratum corneum because the nuclei disappear when cells move above the granular layer.

In the thickened epidermis of psoriasis a greater number of labeled cells is present in the basal layers of the epidermis than is found in normal epidermis (fig. 6). Van Scott and Ekel showed that mitoses are also limited to the lower 3 rows of cells in psoriasis (*3*). Progression of labeled cells as they migrate upwards is seen in figure 7. They reach the top of the viable cell population in psoriasis at 48 hours, as opposed to 12–14 days in normal epidermis, and they have moved through a larger cell population than in normal epidermis (*5*). The marked increase in the turnover rate of psoriatic epidermis is obvious.

The turnover time of the stratum corneum has been measured by use of radioactive amino-acid incorporation into protein that will become part of stratum corneum. The movement of protein can be followed by autoradiographic or biochemical analysis (*2, 6*). A fluorescent compound, tetrachlorsalicylanilide, has also been used to measure the turnover of stratum corneum (*7*). The data from all these studies indicate that the turnover time of normal stratum corneum is about 2 weeks and will vary depending on the site measured and the technique used.

In psoriasis the nuclei of cells are retained in the stratum corneum (parakeratosis) so that labeled cells can be followed as they move upward to the surface. Cells with labeled nuclei are at the surface in 4 days after injection or, in other words, have spent 2 days in transit through the viable stratum spinosum and approximately 2 more days in transit through the stratum corneum (*5*).

It is now necessary to examine cell reproduction in the basal cell compartment, which provides the force for cell migration. Referring to the cell cycle again, those cells labeled during DNA synthesis are counted as they migrate through mitosis at different hours. The first labeled cells completing DNA synthesis will appear as labeled mitoses within a few hours. Thereafter, for the next few hours, all mitoses should be labeled, thus producing the classical labeled mitoses curve (text-fig. 2). From this curve, the duration of DNA synthesis can be determined as the distance between the 37½% points on the ascending and descending limbs of the curve (*8*). In psoriasis, the DNA synthesis period is approximately 8½ hours and the G_2 period is approximately 4 hours (*9*).



TEXT-FIGURE 2.—DNA synthesis in psoriasis. Labeled mitoses curve to determine the durations of the DNA synthesis and G_2 premitotic periods. Curve is drawn through *points,* each of which represents the mean of the percent-labeled mitoses in 8 patients. *Vertical lines* represent 80% confidence limits for each mean.

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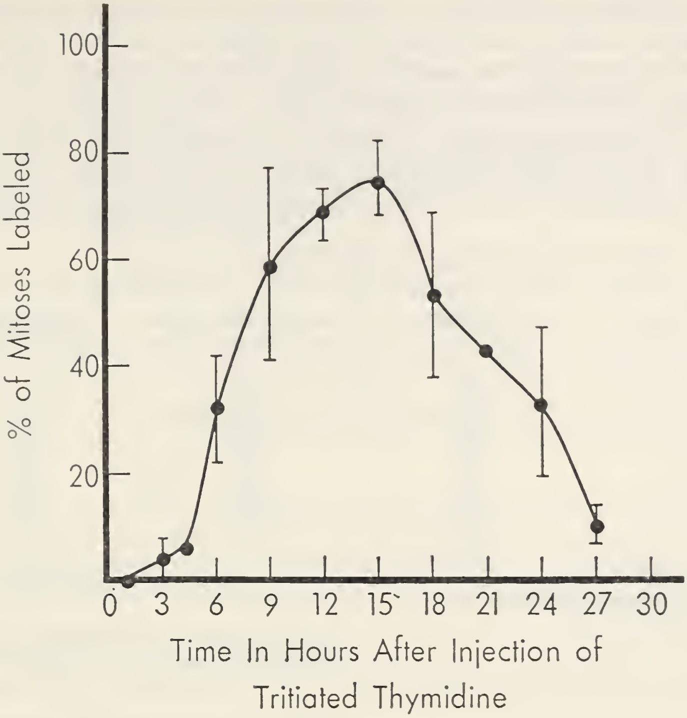
In normal epidermis, which is more difficult to study because of the small number of mitotic figures present, the results from the first 6 patients studied reveal a significantly different DNA synthesis duration from that in psoriasis. The S period in normal epidermis is about 16 hours, compared to 8½ hours in psoriasis, and the G_2 period is a little longer, about 6 or 7 hours (text-fig. 3). Further studies to confirm these data are



Text-figure 3.—DNA synthesis in normal epidermis. Labeled-mitosis curve of normal epidermis. Six patients studied at this time.

being obtained. While some investigators have considered the S period to be a fairly consistent value in many cell populations, a review of the literature shows values ranging from 5–30 hours (table 1). The significant difference in the S period of psoriatic and normal epidermis suggests that the pathophysiology of psoriasis in some way affects DNA synthesis.

Table 1.—Duration of cell cycle ($T_{g.c.}$) and DNA synthesis period (T_s) in various epithelial tissues, *in vivo*

Epithelial tissue	Animal	$T_{g.c.}$ (hr)	T_s (hr)	Reference
Epidermis	Mouse	150	8	(*10*)
Ear epidermis	Mouse	24 days	30	(*11*)
Corneal	Mouse	70	8	(*12*)
Esophagus	Mouse	108	7	(*4*)
Forestomach	Mouse	260	13. 5	(*13*)
Ileum	Mouse	18.7	7. 5	(*14*)
Uterine	Mouse	42	8. 5	(*12*)
Epidermis	Hairless mouse	110	5. 3	(*15*)
Lens	Rat	52–300	10. 5	(*16*)
Vaginal	Rat	13–15	6	(*17*)
Cheek pouch	Hamster	142	10. 2	(*18*)
Epidermis	Human	152	—	(*19*)
Gastrointestinal tract.	Human	24 (minimum)	10–15	(*20*)

To determine the duration of the entire cell cycle, the proportion of proliferative cells in the S period at any time, *i.e.*, the labeling index, is needed. In psoriasis the lowest 2–4 layers of cells contain labeled cells; mitoses are found within this same area (fig. 7). The proportion of labeled cells in the total proliferative population was determined (table 2). In 7 patients with a chronic steady-state plaque form of psoriasis, the labeling index averaged 22.7%. In normal epidermis the labeling index is 5.2%.

TABLE 2.—Labeling index in psoriatic epidermis*

Patient	Labeled cells (N_s)	Germinative cells ($N_{g.c.}$)	Labeling index (% $N_s/N_{g.c.}$)†
A	188	887	21.2
B	160	789	20.3
C	91	335	25.6
D	158	640	24.7
E	164	812	20.2
F	49	225	21.8
G	354	1410	25.0

*The labeling index includes a correction factor of 1.4 to compensate for thickness of histologic preparations and the 1.5–2.0 μ range of β-emissions (*9*).

†Average 22.7%; SD ± 2.4.

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The germinative cell cycle in psoriatic and normal epidermis is calculated by equation [1] to be 37.5 and 308 hours, respectively.

$$\frac{N_s}{T_s} = \frac{N_{g.c.}}{T_{g.c.}} \qquad [1]$$

N_s=number of cells in S phase
T_s=duration of S phase
$N_{g.c.}$=total number of germinative cells
$T_{g.c.}$=duration of entire germinative cell cycle
$N_s/N_{g.c.}$=labeling index

The duration of each part of the cycle is summarized in table 3.

TABLE 3.—Germinative cell cycle in human epidermis*,†

	L.I. (%)	S (hr)	G_2 (hr)	M (hr)	G_1 (hr)	G.C.C. (hr)
Basal cell carcinoma	9.2	20	7	1.5	188	217
Normal‡	5.2	16	6–8	1	284	308
Psoriasis	22.7	8.5	4	0.3	24	37.5

*Summary of the values for the germinative cell cycle and its component parts in psoriatic and normal human epidermis and basal cell carcinoma. The median G_2 period technically includes one half of the mitotic period.

†S = DNA synthesis phase; G_2 = mean interval between S and mitosis; M = duration of mitosis; L.I. = labeling index of germinative cells; G.C.C. = germinative cell cycle.

‡Preliminary data.

Diurnal variations in mitotic index have been reported in animal and human tissues. We have studied normal patients at bedrest for diurnal variation of the labeling index (*21*). There is a slight rise in labeling index at midnight, far below the reported increases of mitotic counts at different hours. For the most meaningful studies, mitotic and labeling indexes alone are not adequate without determining the duration of each of these phases. These indexes can be substantially influenced by changes in *duration* of mitosis or the S period.

Buccal mucosa, another tissue studied in humans, shows labeling in the lower rows of the epidermis. These cells migrate to the surface through the differentiated compartment in approximately 4 days. This is rapid but, in the gastrointestinal tract, turnover time is faster, in the order of 2 days (*20*). This rapid turnover may explain why buccal mucosal ulcerations result from treatment with chemotherapeutic drugs whereas in normal skin, whose rate of turnover is so slow, such manifestations of drug toxicity occur only rarely.

The human hair bulb is probably the fastest reproducing organ in the body. Thymidine labeling an hour after injection is primarily in the lower half of the matrix with a large proportion of the cells being labeled (fig. 8). Van Scott *et al.* have calculated that the turnover of the hair germinative population is less than 24 hours (*22*). Within 48 hours, labeled cells have migrated up to the keratinizing area to form the hair shaft. The high rate of cell proliferation in the hair bulb accounts for its sensitivity to most cancer chemotherapeutic agents.

In conjunction with Dr. H. Averette, we are investigating cell proliferation kinetics of vaginal epidermis under different states of hormonal stimulation (*23*). One hour after injection with tritiated thymidine the vaginal epidermis of a postmenopausal woman showed few labeled cells. After treatment with Premarin or diethylstilbestrol, specimens labeled with thymidine showed a large increase in the number of labeled cells (table 4). Animal experiments by other workers confirm that hormones have a distinct effect on DNA synthesis and cell proliferation in hormonally responsive tissues or "target organs" (*17*, *24*).

TABLE 4.—Labeling index and transit time of human vaginal and cervical epithelium*

	Labeling index		Transit time	
	Number of patients	% ± SD	Number of patients	Hours
Normal periods	6	9.3 ± 2.2	3	96
Postmenopausal, unstimulated	6	5.3 ± 0.2	1	96
Postmenopausal, stabilized estrogen	5	11.3 ± 0.2	1	96
Postmenopausal, hyperstimulated	4	23.2 ± 2.2	3	96

*Stabilizing dose of estrogen is 0.5 mg diethylstilbestrol *per os* daily or 1.25 mg Premarin *per os* daily. Hyperstimulating dose of estrogen is intravaginal application of Premarin cream 2 gm twice daily in addition to the stabilizing dose.

The transit time of thymidine-labeled cells through the vaginal epithelium is, as in buccal mucosa, approximately 4 days regardless of whether the patient is premenopausal or postmenopausal. In postmenopausal women, supplementary estrogens do not appear to influence the transit time (table 4). Further studies are required to elucidate the pattern(s) of cell proliferation in the hormonally sensitive vaginal and cervical epithelium.

Another group of diseases, the ichthyosiform dermatoses, have been reclassified recently into four categories; one of the criteria was the rate of proliferation (*25*, *26*). Lamellar ichthyosis and epidermolytic hyperkeratosis are both characterized by rapid transit of labeled cells through the epidermis. Both of these diseases have heavy, thick, rapidly accumulating scale. In the other two milder forms of ichthyosis, vulgaris and X-linked, cells proliferate at a normal rate (table 5). In addition, all four types of ichthyosis have particular clinical, histological, and genetic characteristics.

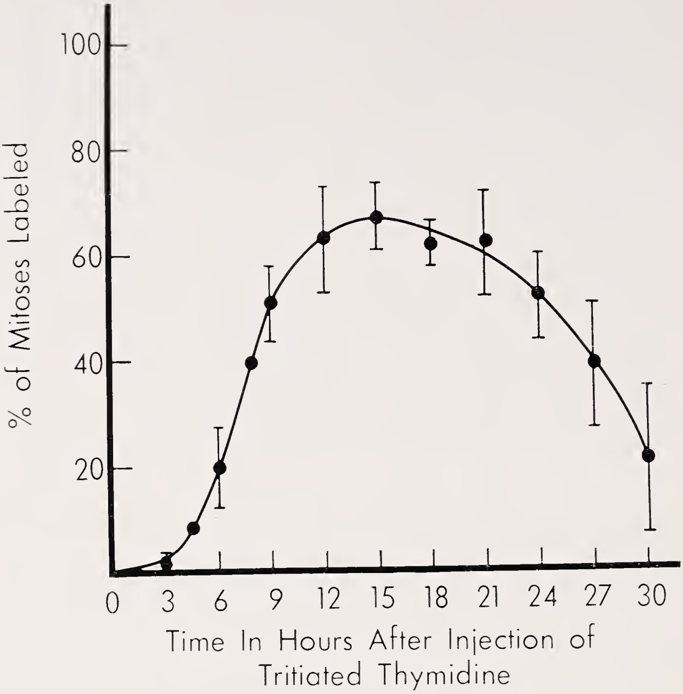
The final group of problems to be discussed are the cutaneous malignancies, both those primary to the skin and those metastatic from internal organs. The basal cell carcinoma is the most common skin tumor and is particularly prevalent in the southern part of the United States. It is a slowly growing tumor that is locally invasive but does not metastasize. It is a unique type of tumor because, histologically, it is characterized by nests of homogeneous cells resembling basal cells of the epidermis, hence the name. One hour after exposure to a local injection of tritiated thymidine, a significant number of labeled cells are scattered randomly throughout the tumor (fig. 9). In 18 patients, tumor specimens from the labeling index were calculated to be 9.2 ± 3.2% (*27*). To measure the labeling index, only areas of tumor uniformly labeled were counted, because in the process of local injection, the tumor may not have been uniformly exposed to tritiated thymidine. The method of analysis used here gives the maximum labeling index present in these tumors. The DNA synthesis period was determined by the techniques described above to be 20 hours in 9 patients with basal cell carcinoma (text-fig. 4). This S period is significantly longer than in normal epidermal basal cells and much longer than in psoriasis.

The results indicate that basal cell carcinoma cells have a cell cycle of 217 hours; comparisons of cell cycle times in normal and psoriatic skin are given in table 3. Interestingly enough, whereas psoriasis is very responsive to methotrexate, basal cell carcinoma is not, at least when used as described by Van Scott *et al.* (*28*). Since basal cell carcinomas grow exceedingly slowly but their cells are doubling at a much faster rate, we must suggest that cell death occurs at a rate almost equal to cell division.

Very little information is available on cell proliferation in other human malignancies such as those of the lung and breast and in melanoma. We are in the process of studying these tumors in cases where they have

TABLE 5.—Summary of data on cell proliferation in ichthyosiform dermatoses

Condition	Labeled cells/cm, basal line ±S	Mitoses/cm, basal line ±S	Labeled cells/cm, surface line ±S	Mitoses/cm, surface line ±S	Cell transit time through epidermis (days)
Normal controls	68. 9 ± 17	7. 7 ± 3. 3	86. 2 ± 21	8. 7 ± 4. 1	12–14
Ichthyosis vulgaris	49. 3 ± 3. 5	2. 5 ± 1. 8	69. 6 ± 7. 2	3. 1 ± 2. 3	10–14
X-linked ichthyosis	45. 6 ± 15	7. 6 ± 6. 0	54. 6 ± 17. 9	9. 0 ± 7. 3	14–16
Lamellar ichthyosis	134 ± 15	24. 8 ± 6. 7	231 ± 3. 6	30. 7 ± 8. 3	4–5
Epidermolytic hyperkeratosis	251 ± 104	40. 7 ± 16. 1	294 ± 85	55. 0 ± 27. 1	4



TEXT-FIGURE 4.—DNA synthesis in basal cell carcinoma (8 patients).

metastasized to the skin. DNA synthesis curves of the first patients studied are presented in text-figure 5. In these three patients it should be noted that the S period will be quite long.

Therapy of psoriasis with methotrexate can be examined in relation to the data on cell proliferation kinetics. Text-figure 6 compares the cell cycles of psoriatic and normal epidermal cells schematically. When methotrexate is administered, approximately 23% of the germinative basal cells in psoriasis are *selectively* affected whereas a very much lower percentage of normal cells will be attacked. Secondly, a shorter S period with a higher rate of DNA synthesis in psoriasis suggests greater sensitivity to this DNA poison. Based on the results of this study, a therapeutic schedule to be clinically tested will be the administration of methotrexate in three or four divided doses over the life cycle of the psoriatic cell population. The effectiveness of methotrexate demonstrates the selective toxicity of a chemotherapeutic agent in populations of cells with different rates of proliferation (*29*, *30*).

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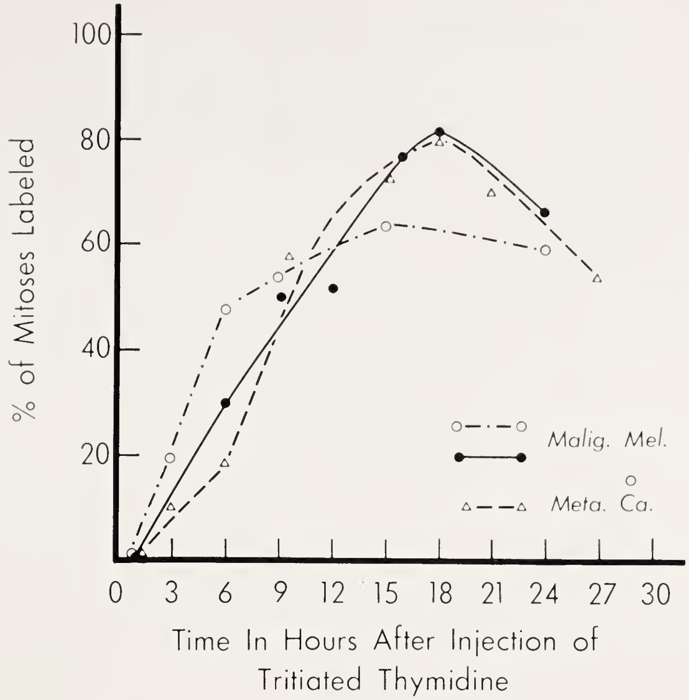

TEXT-FIGURE 5.—DNA synthesis curves for malignant melanoma (2 patients) and lung carcinoma (1 patient) metastatic in the skin.

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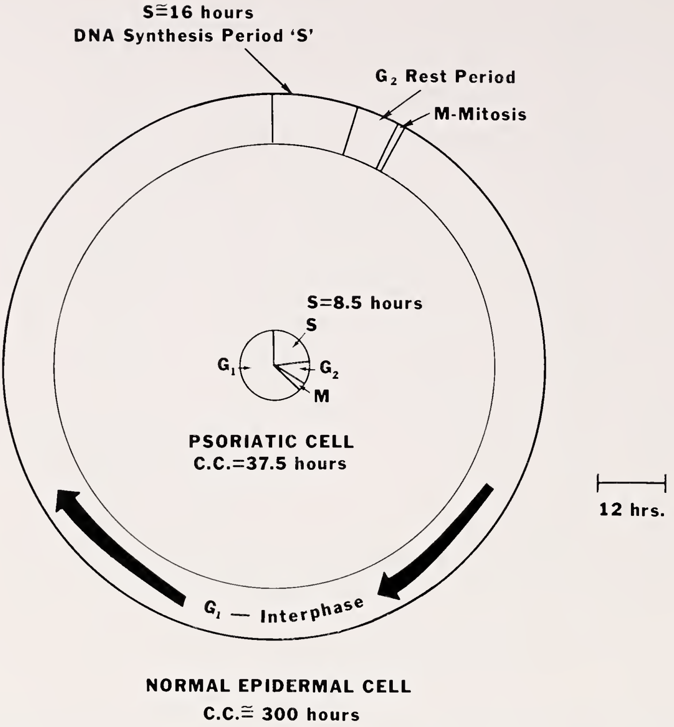

TEXT-FIGURE 6.—Germinative cell cycle of normal and psoriatic epidermal cell drawn to scale to show relative size and duration of each phase.

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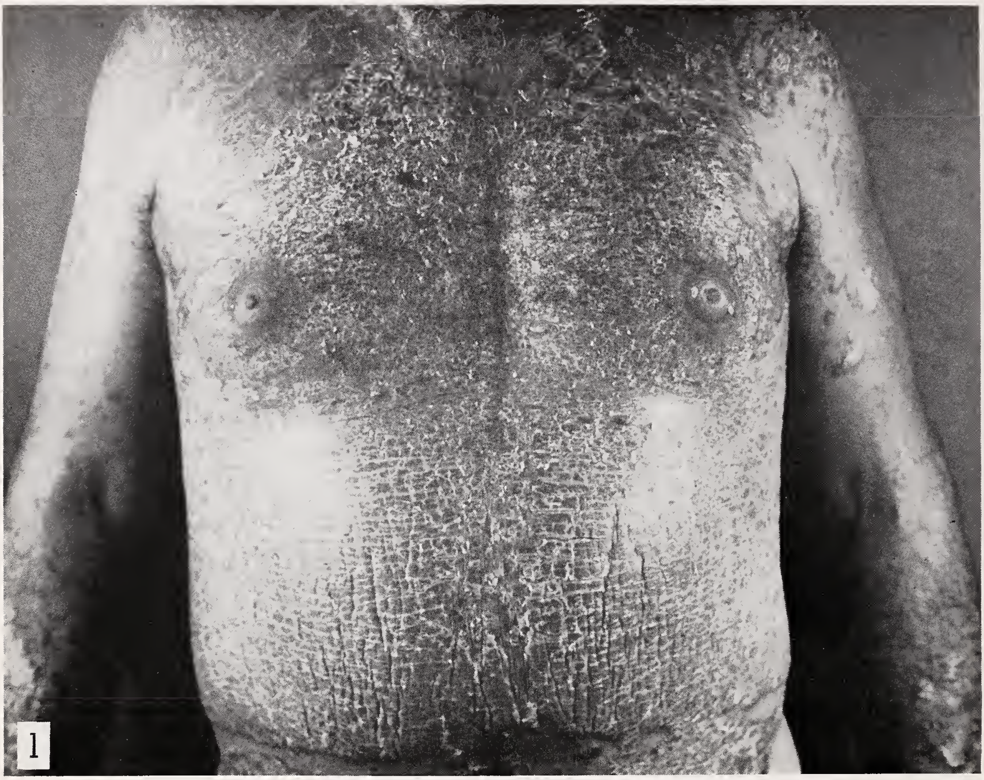

FIGURE 1.—Patient with psoriasis of 20 years' duration, which involves about 80% of his body. Before treatment with methotrexate.

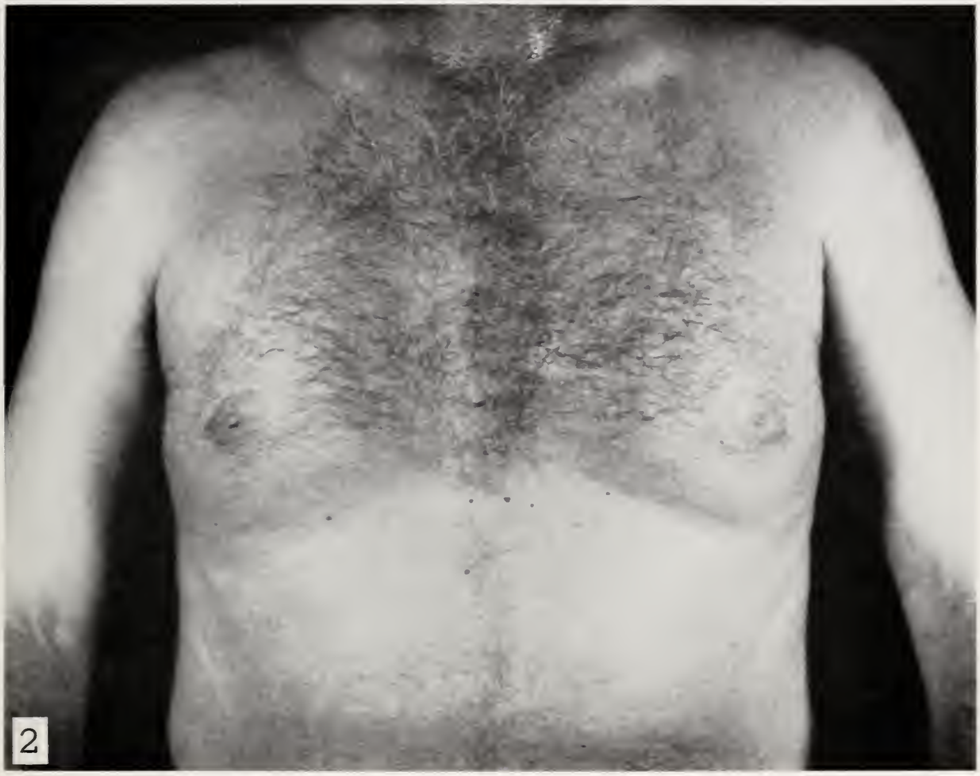

FIGURE 2.—Same patient after 2 months of therapy with methotrexate (50 mg intramuscularly, weekly). No toxicity from the drug was seen.

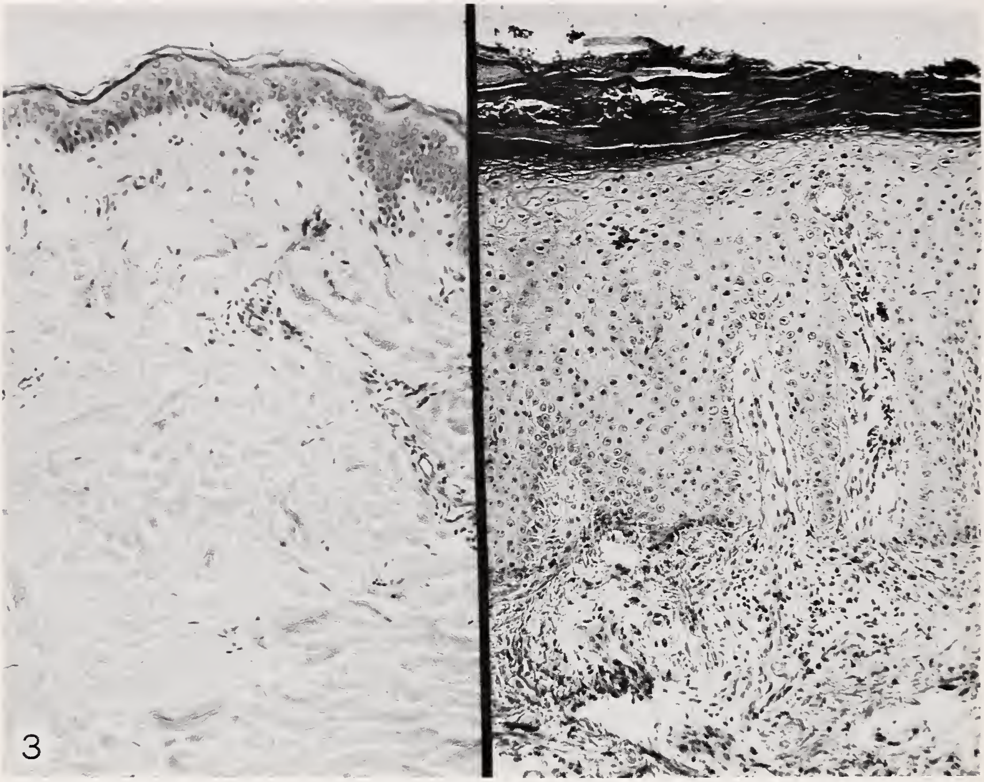

Figure 3.—Typical histologic sections of normal (*left*) and psoriatic skin at the same magnification. *Note* marked thickness of psoriatic epidermis and especially the stratum corneum, which appears clinically as the typical white scale.

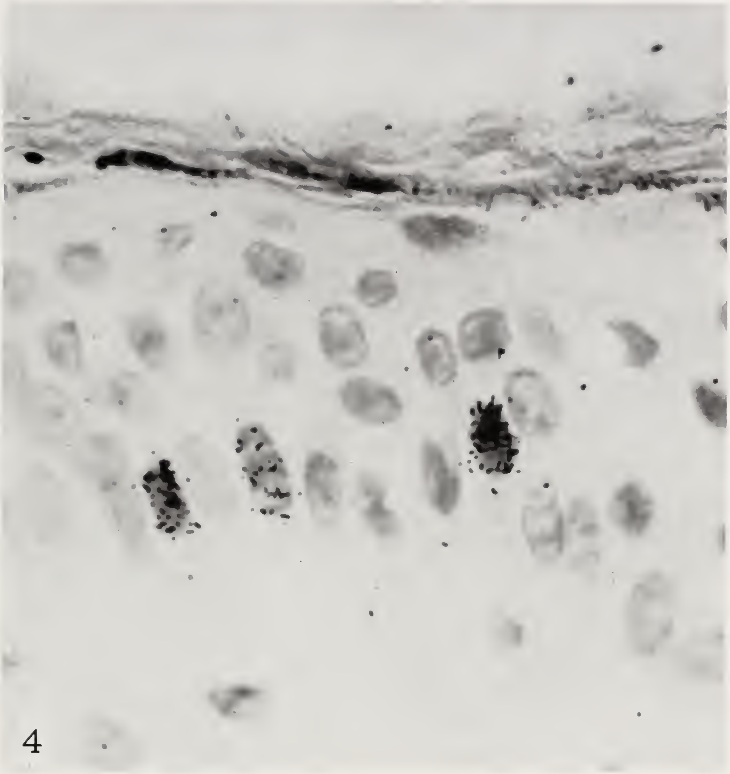

FIGURE 4.—Autoradiograph of normal human epidermis 1 hour after local injection of tritiated thymidine. Epidermal cells labeled in basal cell layer only.

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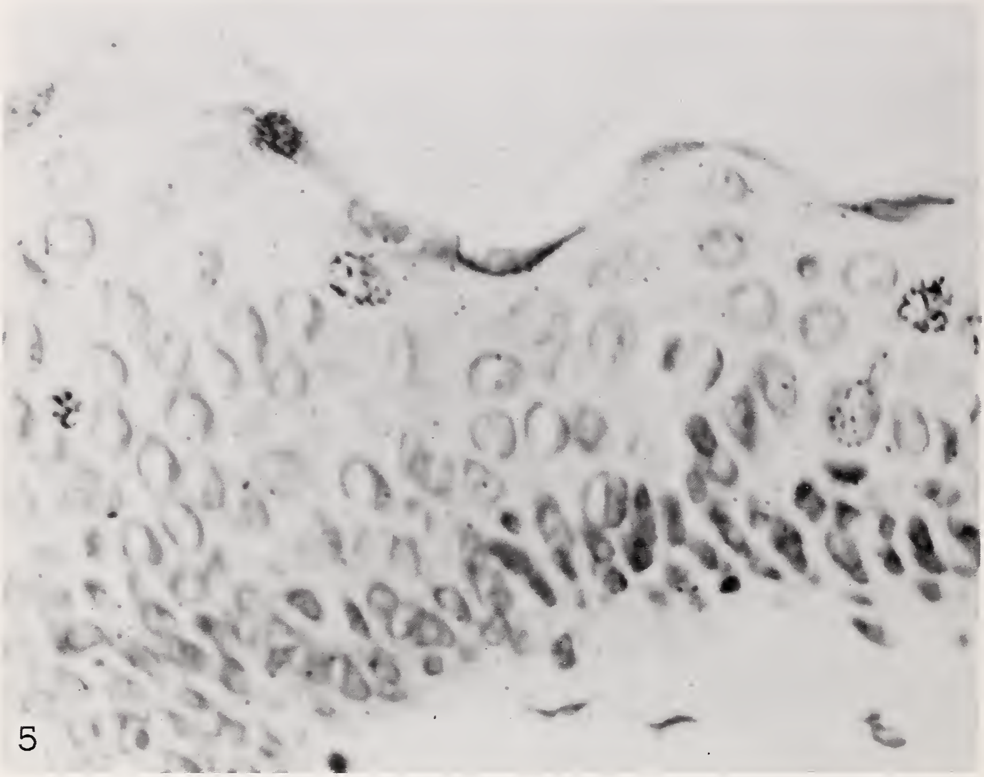

Figure 5.—Autoradiograph 12 days after injection of tritiated thymidine. Labeled cell is now at outer limits of viable differentiated cell compartment.

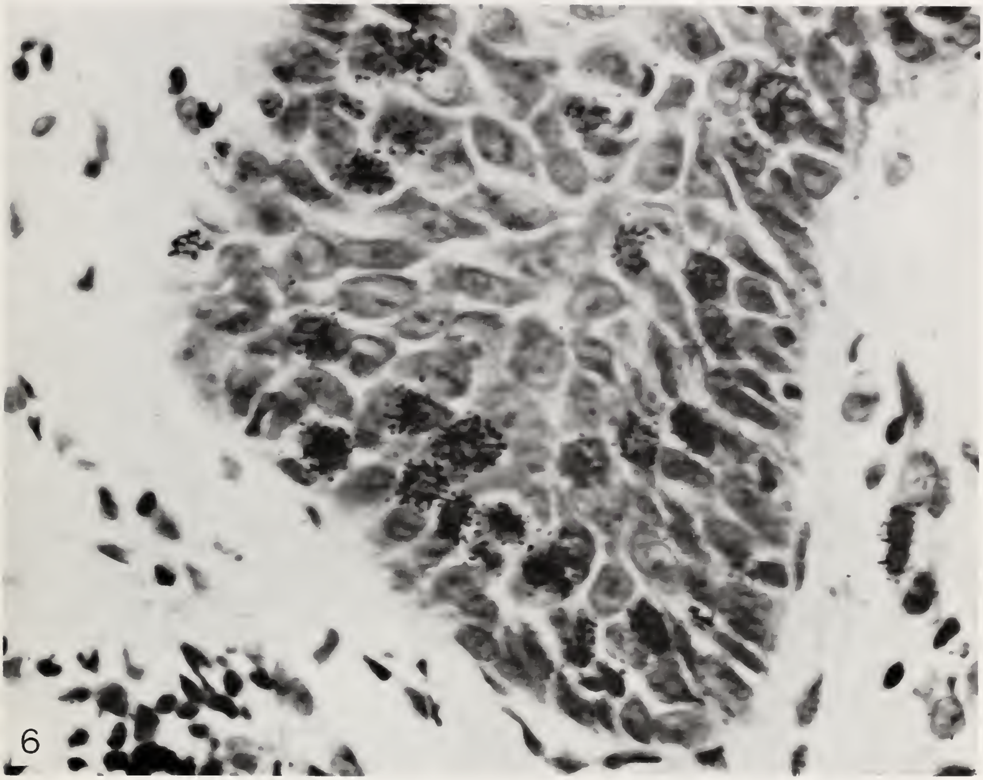

FIGURE 6.—Autoradiograph of psoriatic epidemis 1 hour after local intradermal injection of tritiated thymidine. Nuclear labeling is seen primarily in the first three cell layers of epidermis which constitute the germinative zone.

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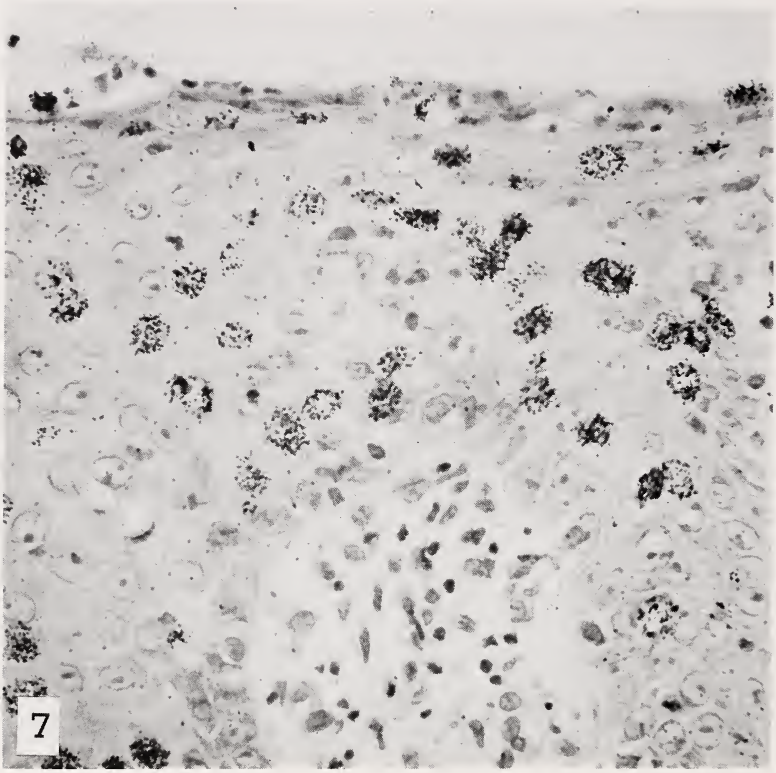

Figure 7.—Autoradiograph of psoriatic epidermis 48 hours after injection of isotope. Labeled cells have reached the outer limits of the stratum spinosum.

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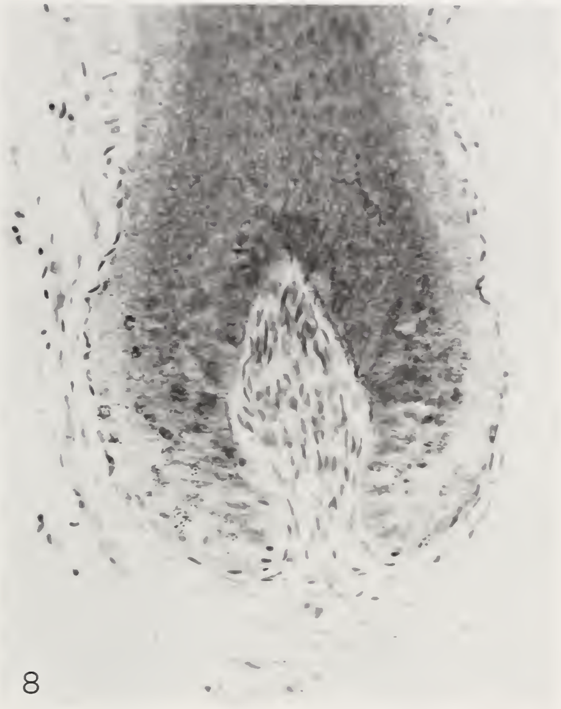

FIGURE 8.—Autoradiograph of human hair matrix 1 hour after intradermal injection of tritiated thymidine. Numerous labeled cells are visible in *lower half* of hair matrix.

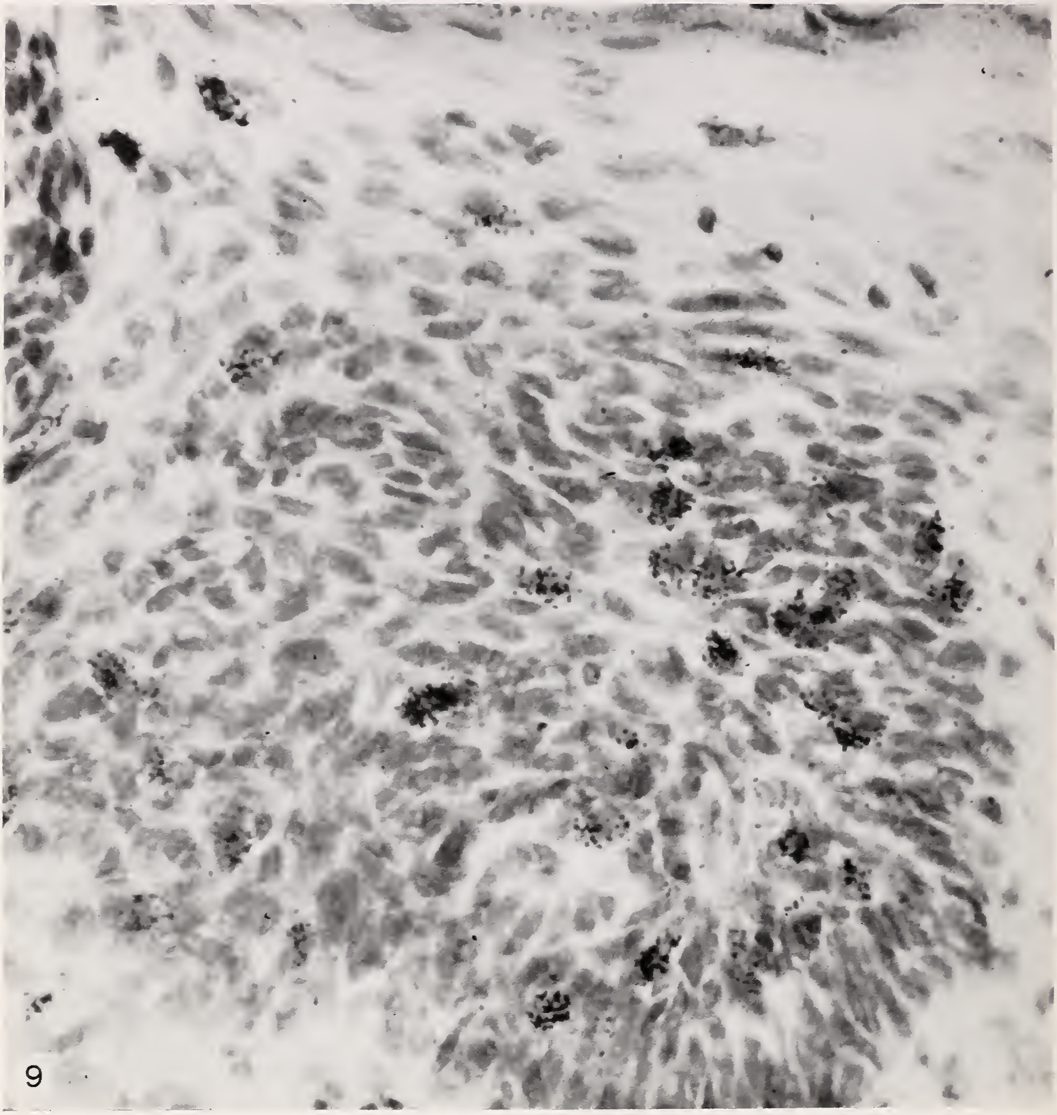

Figure 9.—Basal cell carcinoma, 1 hour after injection of tritiated thymidine, showing labeled cells throughout the tumor.

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